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Treatment of Amoebic Gill Disease (AGD) Caused by *Neoparamoeba Perurans* in Atlantic Salmon (*Salmo Salar*)

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Declarations by the Author

Statement of Originality

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The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

Signed

Shijie Li

Statement of Co-authorship

Statement of Co-Authorship

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Abstract

Neoparamoebae perurans is a marine protozoan parasite that infects gills of cultured Atlantic salmon, *Salmo salar*, causing amoebic gill disease (AGD) which is currently reported in most salmon producing countries. In Tasmania, the management of AGD in Atlantic salmon accounts for 20% of production costs. As a result, several studies have been done to explore alternative treatment methods. However, previous studies other than freshwater bathing focused solely on testing chemotherapeutants against AGD. With the exception of garlic *Allium sativum* and AQUI-S® (product based on clove oil), no other plant products have been tested against AGD.

In the first study, the effects of clove oil and its derivative AQUI-S® against *N. perurans* were studied. *N. perurans* trophozoites were exposed to clove oil at 10, 20, 40 and 80 µL L^{-1} for 10 min and 5, 10, 20 and 40 µL L^{-1} for 120 min; AQUI-S® at 5, 10, 20 and 40 µL L^{-1} for 10 min and 2.5, 5, 10 and 20 µL L^{-1} for 120 min. There were no significant differences in viability and survival of trophozoites after exposure to the anaesthetics. When trophozoites were exposed to clove oil at 80 µL L^{-1} for 10 min there was significantly ($P < 0.05$) more detachment from a plastic surface compared to the control. Continued use of clove oil and AQUI-S® at 40 µL L^{-1} or at lower concentrations for up to 120 min is unlikely to have a detrimental impact on amoebae that are isolated and collected from salmon with AGD to be used for AGD research.

In the second study, viability estimations using viability assays were compared to manual counting using trypan blue and neutral red vital dyes. The four viability assays examined were CellTiter-Blue®, CellTiter-Glo®, CellTiter-Fluor™ and CytoTox-ONE™. A stock solution

comprising of 50% live cells and 50% dead cells (25% heat inactivated, 25% formaldehyde inactivated) was used. All viability assays and manual counting using neutral red estimated viability of cells in stock solution to be approximately 50%, except trypan blue which overestimated cell viability. A positive linear relationship between the signal generated and the number of live cells was observed when the maximum number of viable cells did not exceed 1500. However, positive linear relationship between signal generated and the number of live cells was only observed in CellTiter-Glo® and CellTiter-Fluor™ when the maximum number of viable cells did not exceed 10000. CellTiter-Glo® was determined to be the most suitable viability assay due to the ease of use and the ability to maintain linearity over a wide range of viable cells.

In the third study, efficacy of GTE (green tea extract) and OLE (olive leaf extract) against *N. perurans* was examined *in vitro* using gill isolated amoebae and exposing them to GTE at 9, 19, 38, 75, 150 and 300 mg L^{-1} and OLE at 12500, 6250, 1562, 780 and 390 mg L^{-1}. OLE and GTE were found to be effective against *N. perurans* at 780 mg L^{-1} and 150 mg L^{-1} respectively. Safety of GTE and OLE to Atlantic salmon were examined using bath treatment of either 1500 mg L^{-1} OLE or 75, 150 & 300 mg L^{-1} GTE in seawater; only OLE was considered safe. Subsequently in the fourth study, efficacy of OLE as bath treatment for AGD was studied by bathing AGD affected salmon at 780 mg L^{-1} for 1 h. OLE significantly reduced the prevalence of fish affected by AGD both immediately after and seven days after bath treatment.

This thesis identified OLE as a potential therapeutic compound against AGD caused by *N. perurans*. This finding serves as a proof of concept that plant products such as OLE could

have therapeutic effects against AGD. This indicates that OLE could be worth examining against other ectoparasitic diseases and OLE warrants further investigations as a potential in-feed treatment for AGD in Atlantic salmon.

Chapter One

General Introduction

Chapter 1 General introduction

1.1 Impact of amoebic gill disease on Atlantic salmon

Amoebic Gill Disease (AGD), caused by *Neoparamoeba perurans*, is the most significant health problem affecting the Tasmanian Atlantic salmon industry (Nowak et al., 2002). In recent years, AGD has become a significant health concern for the marine salmon farming industry in Ireland, Scotland and France (Rodger, 2014). In Tasmania, AGD mitigation cost is estimated to account for 20% of the production cost (Kube et al., 2012). Mortalities as a result of AGD cost Norway US$12.55 million in 2006 and Scotland US$81 million in 2011 (Shinn et al., 2015). The increased cost of production is not only due to disease management, but also loss of fish condition, reduced growth and increased feed conversion ratio (Leef et al., 2007a). If salmon affected by AGD are left untreated, mortalities can reach 50% and lead to an even greater financial loss (Munday et al., 1990). In a severe case, mortalities of 82% was reported (Steinum et al., 2008).

Environmental factors increasing the risk of AGD outbreaks include water temperature and salinity (Clark and Nowak, 1999; Douglas-Helders et al., 2001; Adams and Nowak, 2003). The highest risk is during summer months when water temperature range between 12 and 20 °C (Munday et al., 1990). Other factors thought to affect AGD outbreaks include oxygen levels, husbandry practises, rainfall (Clark and Nowak, 1999), biofouling (Tan et al., 2002), overall fish health (Douglas-Helders et al., 2001) and presence of salmon carcases within production cages (Douglas-Helders et al., 2000).

1.2 Causative agent

N. perurans is a marine protozoan that has been identified as the causative agent of AGD (Crosbie et al., 2012a). *Neoparamoeba pemaquidensis* was regarded as the aetiological agent of AGD of Atlantic salmon (*Salmo salar*) when the disease first emerged. This conclusion was based on the presence of one or more intracellular perinuclear bodies (parasomes) and the lack of surface scales (Kent et al., 1988). However, phylogenetic analyses of the 18S and 28S rRNA gene sequences from isolated *Neoparamoeba* indicated that it was distinctly different from *N. pemaquidensis* and *Neoparamoeba branchiphila*. This amoeba, hence, was a new species and named as *N. perurans* (Young et al., 2007). *N. perurans* was the only amoebae species detected in archival histopathology blocks containing AGD affected gill samples in Tasmania and in four other host species from six countries (Young et al., 2008b).

N. perurans is a free-living facultative ectoparasite that lacks organised surface structures and possesses one or more endosymbionts (parasomes) related to *Ichthyobodo necator* (Dyková et al., 2003). All species of the genus *Neoparamoeba* share similar ultrastructural features, defined plasma membrane, endocytotic vesicles and presence of an endosymbiont (Dyková et al., 2003; Dyková et al., 2005; Dyková et al., 2008). While morphological features distinguish *Neoparamoeba* from other vexilliferids, efforts to define members within this genus using morphology have been unsuccessful (Dykova et al., 2000; Dyková et al., 2005). The only way to identify *N. perurans* from the other species of *Neoparamoeba* is by using molecular methods (Young et al., 2007).

The ability of *N. perurans* to osmoregulate in 28ppt seawater using contractile vacuoles was first described by Lima et al. (2016). When *N. perurans* trophozoites were subjected to hypotonic stress, excess cytosolic water was actively transported out of the cells by contractile vacuoles until the amoeba cytoplasm reached isotonic equilibrium with the surrounding medium (Lima et al., 2016). In addition, 16% of amoebae were able to recover from a 2h long incubation in freshwater by pseudocyst formation (Lima et al., 2017). The ability of *N. perurans* trophozoites to osmoregulate in combination with the formation of pseudocyst during freshwater bathing is of significant relevance as freshwater bathing is the only commercial treatment for AGD infected fish. This explains amoebae survival and rapid reinfection levels following freshwater bathing (Powell and Clark, 2003).

Amoebae would consistently bring along bacteria to the culture when they are isolated from a natural environment (Dyková and Kostka, 2013), although paramoebae utilise bacteria as a food source, their relationship with bacteria is more complicated than just a source of sustenance. During phagocytosis of bacteria, trophozoites are exposed to foreign DNA and this provides opportunities for horizontal gene transfer (Keeling and Palmer, 2008). This contributed to the genomes of other amoebae such as *Acanthamoeba castellanii* where it was inferred that 2.9% of the proteome originated from horizontal gene transfer (Clarke et al., 2013). In other pathogenic eukaryotes, horizontal gene transfer contributed to the virulence of *Blastocystis* sp. (Eme et al., 2017) and pathogenicity of *Pseudocohnilembus persalinus* (Xiong et al., 2015). Despite evidence in other protozoa, it is unclear if horizontal gene transfer had affected the genome or biology of *Neoparamoeba* species (Tanifuji et al., 2017).

A characteristic morphological feature of *Neoparamoeba*, similar to *Paramoeba* and *Janickina* amoebae, is the presence of one or more intracellular endosymbionts (Dykova et al., 2000; Dyková et al., 2003). These endosymbionts were initially known as "parasome" (Dyková et al., 2003) and after observations of similarities between *Neoparamoeba* "parasomes" and *Perkinsiella amoebae*, *Neoparamoeba* "parasomes" were given the name *Perkinsiella amoebae*-like organisms (PLOs) (Dyková et al., 2003, Moreira et al., 2004). Usually when a host contains an intercellular endosymbiont, they are most likely to have a mutually beneficial association (Moran et al., 2008). Phytogenic study has demonstrated that PLOs are vertically transmitted host to host and the relationship between PLOs and *Neoparamoeba* is hereditary (Young et al., 2014). However, any symbiotic relationship between *Neoparamoeba* and its PLOs is unknown (Young et al., 2014). It has been assumed that this relationship is obligatory and mutualistic as *Neoparamoeba* has always kept at least one PLO in close proximity to its nucleus (Dykova et al., 2000; Dyková et al., 2003).

Current knowledge suggests that bacteria associated with *N. perurans* may increase the severity of AGD outbreaks. Molecular analysis indicated that bacteria from the genus *Psychroserpens* were present on the gills of AGD affected Atlantic salmon but not on the gills of unaffected fish (Bowman and Nowak, 2004). The effect of *Psychroserpens* bacteria on AGD could not be further investigated as it was unamenable to *in vitro* culture (Nowak and Archibald, 2018). Using agar to isolate bacteria from gills of Atlantic salmon, *Winogradskyella* sp. bacteria were identified on AGD affected fish but not from naïve fish (Embar-Gopinath et al., 2005). When Atlantic salmon was co-infected with *Winogradskyella* sp. bacteria and *N. perurans* trophozoites, it increased the severity of AGD lesions to 50% of gill filaments affected compared to 18% in fish infected with only trophozoites (Embar-

Gopinath et al., 2005). This increase in AGD severity is not concentration dependent as a higher concentration of bacteria did not result in a greater prevalence of AGD (Embar-Gopinath et al., 2006). In another experimental AGD study, no significant correlation could be identified between *Yersinia ruckeri* infection before seawater transfer and AGD severity post seawater transfer (Valdenegro-Vega et al., 2015). Currently, there is no evidence to prove that bacterial infection or the presence of non-pathogenic bacteria may increase the severity of AGD (Nowak and Archibald, 2018).

Amoebozoa have been assumed to be asexual based on lack of observations (Hofstatter et al., 2018). This assumption however is questionable due to the difficulty in culturing and observing microbial eukaryotes (Oldham et al., 2016). Recently published data have indicated the presence of meiotic genes in Amoebozoa proving that amoebozoans are ancestrally and "secretly" sexual (Tekle et al., 2017). Furthermore, there has been suggests that most amoebozoans may perform mixis, recombination, and ploidy reduction through canonical meiotic processes (Hofstatter et al., 2018). Despite evidence indicating the possibility of sexual cycles, sex has not been confirmed nor direct evidence documented in *Neoparamoeba* spp. (Lahr et al., 2011). Therefore, the complete lifecycle and reproduction characteristics of *N. perurans* are still undetermined.

1.2.1 Physiology of AGD

AGD was originally thought to induce respiratory failure as both lethargy and respiratory distress have been observed in AGD-affected salmonids (Kent et al., 1988; Munday et al., 1990). However, this was questioned as only minor differences were observed in oxygen uptake between naïve and AGD-affected fish (Powell et al., 2000; Fisk et al., 2002; Leef et

al., 2005a). Under normoxic conditions, AGD affected fish have normal oxygen uptake rate despite having lowered blood oxygen partial pressure (Powell et al., 2000). Fish were able to maintain oxygen transport through physiological means such as increased blood flow and gill perfusion (Powell et al., 2000).

AGD is also associated with altered cardiac function as infected fish have an overall thickening of the muscularis compactum in the ventricle (Powell et al., 2002b) and vascular hypertension (Powell et al., 2002a). The increased pressure in dorsal and ventral aortic blood (Powell et al., 2002a) may be due to compensatory cardiac remodelling as evidenced by changes in ventricle length and ventricular muscle (Powell et al., 2002b). Atlantic salmon affected by AGD were shown to have increased systemic vascular resistance and lowered cardiac output when compared to other salmonid species (Leef et al., 2005b). Significant cardiovascular effects such as vaso-constriction and hypertension are associated with AGD although the source of these effects is yet to be identified (Powell et al., 2002a; Leef et al., 2007b).

1.2.2 Pathology and Diagnosis

Clinically, AGD-affected fish present with gross gill lesions that are characterised by focal or multifocal raised white mucoid patches and profuse mucus production (Clark and Nowak, 1999; Adams and Nowak, 2001). At production farms, diagnosis of this disease is often done through visual observation of the white mucoid patches present on the gills of infected fish (Taylor et al., 2009). Based upon the prevalence and density of these gross gill lesions, fish are assigned a gill score (Table 1.1). However, gill scores are not universal and fish are scored differently between production farms (Adams and Nowak, 2004; Taylor et al., 2009).

The prevalence and density of these gross gill lesions are also used as a tool to monitor the severity and manage treatment frequencies (Clark and Nowak, 1999; Clark et al., 2003; Adams et al., 2004). Other than gross gill lesions, the clinical manifestation of AGD includes anorexia and increased ventilation rates (Kent et al., 1988; Munday et al., 1990). It is important to note that the presence of gross gill lesions is not only due to AGD and diagnosis based on observation of gill lesions should be only regarded as a presumptive diagnosis (Clark et al., 1997; Adams et al., 2004).

Table 1.1. Gross gill scoring guide for Amoebic Gill Disease (AGD) used by Tassal operations Pty Ltd (Taylor et al., 2009).

Gill Score	Description
0	No signs of infection, gills appear healthy
1	1 white spot, light scarring or undefined necrotic streaking
2	2–3 spots or small mucus patch
3	Thickened mucus patch or grouping of spots of ~20% of gill area
4	Lesions covering ~ 50% of gill area
5	Lesions covering most of the gill surface

The most common histological feature of AGD is multi-focal hyperplasia of the lamellar epithelium. The hyperplasia causes fusion of secondary lamellae, formation of inter-lamellar vesicles, reduction of chloride cells and an increase in the number of mucus cells (Powell et al., 2001; Adams and Nowak, 2003). Histological examination of gills has been a reliable method of diagnosing AGD (Clark and Nowak, 1999). Diagnosis is achieved when

trophozoites with one or more parasomes are found in close proximity of hyperplastic gill lesions (Dyková and Novoa, 2001).

Diagnosis of AGD using molecular biology methods can be achieved by either real-time TaqMan® PCR (Downes et al., 2015) of gill samples from fish with suspected AGD, PCR of gill swabs obtained from gross gill lesions (Young et al., 2008a; Bridle et al., 2010) or *in situ* hybridization of histological sections using 18S rRNA oligonucleotide probes (Young et al., 2007). Although observation of white mucoid patches has been shown to be a good indicator of AGD when checks are performed by experienced personnel (Clark and Nowak, 1999; Adams et al., 2004; Bridle et al., 2010), this is only suitable for presumptive diagnosis of AGD in regions where AGD is enzootic. Molecular techniques are useful when performing epidemiological studies of outbreaks in areas where AGD is not known to be endemic.

1.3 Current AGD treatment

The current commercially adopted method of AGD management in Tasmania is freshwater bathing. Non-lethal fish sampling is done on a scheduled basis by farms in Tasmania for the gross examination of the gills in order to quantify white mucoid patches. Using Table 1.1 as a guide, fish are given a gill score and based on this gill score it is decided if freshwater bathing is required. Freshwater bathing involves transferring fish from their original pen into a tarpaulin liner filled with freshwater for two to four hours. Recently, a Tasmanian salmon producer has started using well-boat technology for freshwater treatment of fish (Aqua, 2014). Freshwater bathing is also used in Ireland (Downes et al., 2015) and Norway (Powell and Kristensen, 2014). However, it is too costly and/or unfeasible in other AGD affected areas due to the lack of freshwater (Rodger, 2014). In Scotland, Ireland and Norway,

hydrogen peroxide has been successfully used to control AGD in Atlantic salmon (Oldham et al., 2016). Hydrogen peroxide dosage of between 1000 – 1400 mg L^{-1} for 18-22 minutes was employed with success at low temperatures. Yet, when the temperature was above 13.5 °C or fish have gill score of 3 or higher (Table 1.1), hydrogen peroxide is not safe to be used on infected fish (Rodger, 2014).

During freshwater bathing, the normally stable iso-osmotic mucus layer on the gills of seawater reared Atlantic salmon is rapidly hydrated (Roberts and Powell, 2008). This serves to lower gill mucus viscosity and osmolality whilst increasing the shedding of the mucus layer. Gill associated amoebae are then susceptible to the hypo-osmotic effect of freshwater resulting in their detachment and lysis. Disease expression is quickly curtailed following the removal of the amoebae (Adams and Nowak, 2004; Adams et al., 2012).

During the late 1980s when freshwater bathing was first introduced, the entire marine production phase only required 2-3 baths to manage AGD (Foster and Percival, 1988; Clark and Nowak, 1999). In 1999 and 2000, as many as 10 freshwater baths per cage were required to prevent mortalities due to AGD (Powell and Clark, 2002). Parsons et al. (2001b) demonstrated that amoebae were able to survive a 2 hour freshwater bath and the number of amoebae on gills returned to pre-bath numbers 10 days after treatment (Clark et al., 2003). Furthermore, fish were able to be reinfected by *N. perurans* post freshwater bathing without any addition of amoebae, this suggests that some amoebae were able to survive freshwater treatment (Gross et al., 2004). The efficacy of freshwater treatment is variable depending on water quality (Parsons et al., 2001a). Soft freshwater containing between 19.3-37.4 mg L^{-1} $CaCO_3$ have better treatment efficacy compared to hard freshwater

containing between 173-236.3 mg L^{-1} $CaCO_3$ (Powell and Clark, 2003; Roberts and Powell, 2003).

1.3.1 AGD mitigation

Adaptations in husbandry practise could also be used as an AGD mitigation tool. There was a significant negative relationship between AGD prevalence and the number of times the cage net was changed (Clark and Nowak, 1999). Evidence indicates that biofouling may be a reservoir of *N. perurans* and biofouling should be considered as a risk factor for AGD outbreaks (Tan et al., 2002). Pens with heavy biofouling or lower water exchange had more cases of clinically significant AGD (Rodger, 2014). Regular net changes also improve the oxygen levels and water flow within the pens, leading to better farming conditions (Douglas-Helders et al., 2001). Another known reservoir of amoeba is mortalities (dead Atlantic salmon) found within cages. Amoebae remained viable on infected gills for up to 30 hours after fish death and amoebae from dead infected fish is able to colonise previously uninfected dead fish (Douglas-Helders et al., 2000).

Other than the effects of biofouling on AGD, stocking density is also a factor of AGD progression. Increased production volume and higher stocking density have been put forward as risk factors for AGD (Nowak, 2007). When salmon were subjected to experimental AGD challenge, tanks stocked at 5 kg m^{-3} experienced mortalities 6 days earlier than tanks stocked at 1.7 kg m^{-3} (Crosbie et al., 2010). Production cages with higher stocking density were associated with an increased prevalence of AGD (Douglas-Helders et al., 2004). As a result, a commercial producer imposed a lower stocking density in summer in order to reduce AGD prevalence (Nowak, 2012).

In addition to stocking density, AGD is reportedly more difficult to treat as the disease advances (Rodger, 2014). By rotating between fallowed sites, fish required fewer freshwater treatments throughout the production cycle (Douglas-Helders et al., 2004). Additionally, incorporation of a diet that promotes gill health is able to reduced mortalities associated with AGD (Nexus, 2015). More recently, AGD outbreaks were reduced when snorkel cages (10m deep barrier) were adapted for AGD mitigation by filling snorkels with freshwater (Wright et al., 2017).

Despite extensive research on immunostimulants and vaccines against AGD, they were unable to improve the survival of fish with AGD (Nowak et al., 2014; Valdenegro-Vega et al., 2015). The adaptive AGD resistance trait is one of the primary selection traits in the breeding program as it has the highest hereditability and offers the best financial incentive (Kube et al., 2012). Selective breeding of salmon over a 10 year period increased the duration between freshwater bathing by 32% (Evans et al., 2015). Not only could AGD resistance be enhanced by selective breeding, heterosis for AGD resistance was also significant with Atlantic salmon and brown trout *S. trutta* hybrid showing a 48% improvement in AGD resistance when compared against purebred Atlantic salmon (Maynard et al., 2016).

1.3.2 In vitro viability testing

In vitro viability testing is often the first step in the development of any therapy for the treatment of AGD. Thus far, *in vitro* viability testing methods used for *N. perurans* included: vital dye staining after exposure (Powell and Clark, 2004; Peyghan et al., 2008; Florent et al., 2010; Adams et al., 2012), growth on agar media with chemotherapeutant (Howard and

Carson, 1993; Howard and Carson, 1994) and growth on agar media after exposure to chemotherapeutant (Howard, 2001). Viability testing using neutral red vital dye staining involved incubation of cells with the dye, followed by washing and manual cell-counting using a haemocytometer (Adams et al., 2012). When cell viability was determined using growth on agar media, cell suspension was inoculated onto agar plates and incubated for seven days before counting (Howard and Carson, 1993). Despite these methods being used successfully on *N. perurans*, they are time consuming, labour intensive and subjected to human error.

As a result, there is a need to identify a suitable viability assay for *in vitro* viability assessment. There are several commercial assays available, however, these assays were mainly developed for use with mammalian cell lines (Hannah et al., 2001; Monteiro-Riviere et al., 2009; Niles et al., 2009), bacteria (Pang et al., 2010; Pilsczek et al., 2010; Zou et al., 2011; Van den Driessche et al., 2014) or fungi (Ivanova and Uhlig, 2008; Stylianou et al., 2014). Despite that, CellTiter96®, Presto Blue® and Alamar Blue® have been successfully used as viability assays for *in vitro* assessment of antimicrobials against *Acanthamoeba* (McBride et al., 2005; Heredero-Bermejo et al., 2013; Sifaoui et al., 2013). In order for the viability assays to work successfully with *Acanthamoeba*, individual assay needs to be optimised and adapted for use with different strains or species due to possible difference in metabolic activity resulting from different culture conditions (Heredero-Bermejo et al., 2013).

1.3.3 Alternative treatment methods (experimental)

Freshwater bathing is a highly effective treatment for AGD in Atlantic salmon, however it is labour and cost intensive (Munday et al., 2001). A large selection of antibiotics, disinfectants and detergents have been studied *in vitro* (Table 1.2) and *in vivo* (Table 1.3) as either bath or feed additives (Mitchell and Rodger, 2011). Despite significant efforts, the development of an alternate chemotherapeutic solution was unsuccessful due to host toxicity or lack of efficacy and high cost (Alexander, 1991; Howard and Carson, 1994; Mitchell and Rodger, 2011). Bithionol showed both bath and oral treatment efficacy against AGD (Florent et al., 2007b; c; Florent et al., 2009b; Florent et al., 2010). Florent et al. (2007c) demonstrated that seawater bath containing 1 mg L^{-1} bithionol had the same efficacy as freshwater at reducing the prevalence of lesioned gill filaments. Furthermore, oral treatment of bithionol at 25 mg kg^{-1} for two weeks prior and continuing for 4 weeks after *N. perurans* exposure delayed the onset and reduced the prevalence of lesioned gill filaments by 10 d and 53 % respectively (Florent et al., 2007b).

In order to better manage AGD, oral treatment needs to be developed (Parsons et al., 2001b; Munday and Zilberg, 2003). The advantages of oral treatment include reduced animal handling, reduction of labour and infrastructure costs.

Table 1.2. Compounds that have exhibited *in vitro* activity against *N. perurans*. This list only included successful treatments. Minimum inhibitory concentration is abbreviated as MIC.

Classification	Chemical	Delivery route	Dosage	Duration	Efficacy	Reference
Anthelmintic	Bithionol Bithionol sulphoxide	Sw bath	5-10 mg L^{-1}	72 h	100% of amoebae died 99% of amoebae died	Florent et al. (2010)
Anthelmintic	Levamisole	Added to agar growth media	NA	7 d	MIC ≥5µg/ml	Howard and Carson (1994)
Anthelmintic	Levamisole	Added to agar growth media	NA	7-14 d	MIC ≥10µg/ml	Howard and Carson (1993)
Anthelmintic	Napthalagous	Added to agar growth media	NA	7-14 d	MIC ≥15µg/ml	Howard and Carson (1993)
Antibiotic	Nifurpirinol	Added to agar growth media	NA	7-14 d	MIC of ≤5µg/ml	Howard and Carson (1993)
Antibiotic	Quinolone	Added to agar growth media	NA	7-14 d	MIC ≥10µg/ml	Howard and Carson (1993)
Antibiotic	Quinolone	Added to agar growth media	NA	7 d	MIC ≥20µg/ml	Howard and Carson (1994)
Antibiotic	Quinoline	Added to seawater	30µg/ml	4 h	48.47% reduction of viable amoebae	Howard (2001)
Antibiotic	Nitrothiazole	Added to agar growth media	NA	7-14 d	MIC ≥15µg/ml	Howard and Carson (1993)
Antiprotozoal	Mefloquin hydrochloride	Added to agar growth media	NA	7-14 d	MIC of ≤5µg/ml	Howard and Carson (1993)
Antiprotozoal	Chloro-iodo-hydroxyquinoline	Added to agar growth media	NA	7-14 d	MIC of ≤5µg/ml	Howard and Carson (1993)

Classification	Chemical	Delivery route	Dosage	Duration	Efficacy	Reference
Antiprotozoal	8- hydroxyquinoline	Added to agar growth media	NA	7-14 d	MIC of ≤5µg/ml	Howard and Carson (1993)
Antiprotozoal	8-hydroxyquinoline	Added to agar growth media	NA	7 d	MIC ≥10µg/ml	Howard and Carson (1994)
Antiprotozoal	8- hydroxyquinoline (copper salt)	Added to agar growth media	NA	7 d	MIC ≥15µg/ml	Howard and Carson (1994)
Antiprotozoal	5-chloro-7-iodo-8-hydroxyquinoline	Added to agar growth media	NA	7 d	MIC ≥10µg/ml	Howard and Carson (1994)
Antiprotozoal	pyrimethamine	Added to agar growth media	NA	7 d	MIC ≥5µg/ml	Howard and Carson (1994)
Antiprotozoal	5-hydroxy-1,4, naphthoquinone	Added to agar growth media	NA	7 d	MIC ≥5µg/ml	Howard and Carson (1994)
Antiprotozoal	Fumagillin	Added to agar growth media	NA	7-14 d	MIC ≥10µg/ml	Howard and Carson (1993)
Antiprotozoal	Pyrimethamine	Added to agar growth media	NA	7-14 d	MIC ≥15µg/ml	Howard and Carson (1993)
Antiprotozoal	Quinine sulphate	Added to agar growth media	NA	7-14 d	MIC ≥20µg/ml	Howard and Carson (1993)
Antiprotozoal	Quinacrine hydrochloride	Added to agar growth media	NA	7-14 d	MIC ≥20µg/ml	Howard and Carson (1993)
Antiprotozoal	Nitroimidazole	Added to agar growth media	NA	7-14 d	MIC ≥20µg/ml	Howard and Carson (1993)
Antiprotozoal	Chloro-iodo-hydroxyquinoline	Added to seawater	20µg/ml	4 h	>90% growth inhibition	Howard and Carson (1993)
Antiprotozoal	8-Hydroxyquinoline	Added to seawater	30µg/ml	4 h	99.47% reduction of viable amoebae	Howard (2001)
Antiprotozoal	Pyrimethamine	Added to seawater	30µg/ml	4 h	82.14% reduction of viable amoebae	Howard (2001)

Classification	Chemical	Delivery route	Dosage	Duration	Efficacy	Reference
Antiprotozoal	Quinacrine hydrochloride	Added to agar growth media	NA	7 d	MIC ≥10µg/ml	Howard and Carson (1994)
Antiprotozoal	5-chloro-7-iodo-8-hydroxyquinoline	Added to seawater	30µg/ml	4 h	72.85% reduction of viable amoebae	Howard (2001)
Coccidiostat	Sulphaquinoxaline	Added to seawater	30µg/ml	4 h	33.3% reduction of viable amoebae	Howard (2001)
Coccidiostat	Sulphaquinoxaline	Added to agar growth media	NA	7-14 d	MIC ≥20µg/ml	Howard and Carson (1993)
Coccidiostat	Narasin	Added to agar growth media	NA	7-14 d	MIC ≥15µg/ml	Howard and Carson (1993)
Coccidiostat	Quinacrine	Added to seawater	30µg/ml	4 h	75.63% reduction of viable amoebae	Howard (2001)
Coccidiostat	Narasin	Added to agar growth media	NA	7 d	MIC ≥20µg/ml	Howard and Carson (1994)
Disinfectant	Hydrogen peroxide	Sw bath	1000 mg L^{-1}	10 min	85% of amoebae died	Adams et al. (2012)
Disinfectant	Hydrogen peroxide	Fw bath	100 mg L^{-1}	1 h	0% survival relative to seawater control	Powell and Clark (2003)
Disinfectant	Chloramine-T	Fw bath	25, 50 mg L^{-1}	3 h	2% survival relative to seawater control	Powell and Clark (2003)
Disinfectant	Ethanol	Added to agar growth media	NA	7-14 d	MIC 10g/ml	Howard and Carson (1993)
Disinfectant	Zephiran	Added to seawater	30mg/L	4 h	>50% growth inhibition	Howard and Carson (1993)
Disinfectant	Chlorine dioxide	Sw bath	10, 25, 50 mg L^{-1}	1/2/3 h	22% survival relative to seawater control	Powell and Clark (2003)
Disinfectant	Zephiran	Added to seawater	30µg/ml	4 h	98.4% reduction of viable amoebae	Howard (2001)

Disinfectant	Chloramine-T	Added to seawater	30µg/ml	4 h	74.4% reduction of viable amoebae	Howard (2001)
Disinfectant	Ethanol	Added to seawater	10g/ml	4 h	66.4% reduction of viable amoebae	Howard (2001)

Table 1.3. *In vivo* testing of compounds from Table 1.2 that have exhibited *in vitro* activity against *N. perurans*. This list included both successful and unsuccessful treatments. Minimum inhibitory concentration is abbreviated as MIC.

Classification	Chemical	Administration	Dosage	Duration	Efficacy	Reference
Antiprotozoal	Fumagillin	In feed	0.1% feed	23 d	Not successful	Alexander (1991)
		In feed	0.3% feed	6 d	Not successful	
Antiprotozoal	Quinacrine	In feed	50 mg kg^{-1} fish d^{-1}	22 d	Not successful	Alexander (1991)
			50 mg kg^{-1} fish d^{-1}	29 d	Not successful	
Antiprotozoal	Albendazole	In feed	10 mg kg^{-1} fish d^{-1}	23 d	Not successful	Alexander (1991)
			50 mg kg^{-1} fish d^{-1}	23 d	Not successful	
Antiprotozoal	Quinacrine	Added to seawater bath	30 ppm	4 h	Not successful	Alexander (1991)
Anthelmintic	Levamisole	Added to freshwater bath	1.25, 2.5, 5 mg L^{-1}	3 h	Reduced mortality post treatment	Zilberg et al. (2000)
Anthelmintic	Bithionol	Added to seawater bath	1 mg L^{-1}	1 h	Reduced percent gill filaments with lesions by 33%	Florent et al. (2007c)
Anthelmintic	Bithionol	In feed	25 mg kg^{-1} feed	2 w	Delayed and reduced the intensity of AGD pathology	Florent et al. (2009b)

Classification	Chemical	Administration	Dosage	Duration	Efficacy	Reference
Anthelmintic	Bithionol	In feed	25 mg kg^{-1} feed	2 w	Reduced percent gill filaments with lesions by 53%	Florent et al. (2007b)
Anthelmintic	Mebendazole	Added to seawater bath	1 ppm	4 h	Not successful	Alexander (1991)
Anthelmintic	Levamisole	Added to freshwater bath	1.25, 2.5, 5 mg L^{-1}	3 h	Increased resistance to reinfection	Findlay et al. (2000)
Antibiotic	Rom/TMP	In feed	22 mg kg^{-1} fish d^{-1}	23 d	Not successful	Alexander (1991)
			50 mg kg^{-1} fish d^{-1}	23 d	Not successful	
			42 mg kg^{-1} fish d^{-1}	10 d	Not successful	
			50 mg kg^{-1} fish d^{-1}	10 d	Not successful	
Coccidiostat	Toltrazuril	Added to seawater bath	10 ppm	4 h	Not successful	Alexander (1991)
Coccidiostat	Amprolium	In feed	50 mg kg^{-1} fish d^{-1}	23 d	Not successful	Alexander (1991)
Disinfectant	Chloramine-T	Added to seawater bath	10 mg L^{-1}	1 h	Similar to freshwater bath	Harris et al. (2004)
Disinfectant	Chloramine-T	Added to seawater bath	10 mg L^{-1}	1 h	Similar to freshwater bath	Harris et al. (2005b)
Disinfectant	Chloramine-T	Added to freshwater bath	10-50 mg L^{-1}	3 h	50% reduction of amoebae numbers	Powell and Clark (2004)
Disinfectant	Chlorine dioxide	Added to freshwater bath	25-50 mg L^{-1}	3 h	50% reduction of amoebae numbers	Powell and Clark (2004)
Disinfectant	Hydrogen peroxide	Added to seawater bath	1250 mg L^{-1}	15min	Similar to freshwater bath	Adams et al. (2012)
Disinfectant	Formalin	Added to seawater bath	0.2 ppm	4 h	Not successful	Alexander (1991)
		Added to seawater bath	100 ppm	4 h	Not successful	
Fungicide	$CuSO_4$ (in citric acid)	Added to seawater bath	2 ppm	4 h	Not successful	Alexander (1991)

Classification	Chemical	Administration	Dosage	Duration	Efficacy	Reference
Fungicide	Malachite green	Added to seawater bath	0.1 ppm	4 h	Not successful	Alexander (1991)
		Added to seawater bath	50 ppm	4 h	Not successful	
Mucolytic	L-cysteine ethyl ester (LCEE)	In feed	52.7 mg kg^{-1} fish d^{-1}	14 d	Delayed progression of AGD	Roberts and Powell (2005b)
Surfactant	Tween 20	Added to seawater bath	100 ppm	4 h	Not successful	Alexander (1991)
Surfactant	Alkadet	Added to seawater bath	25 ppm	4 h	Not successful	Alexander (1991)

1.4 Plant extracts in aquaculture

Applications of therapeutic and prophylactic drugs are widespread in aquaculture production (Rico et al., 2013). Long-term usage of chemotherapeutants results in drug resistance (Miranda and Zemelman, 2002; Seyfried et al., 2010) and potential residues in fish products (Cabello, 2006; Ormazábal et al., 2012). These side-effects have shifted the research focus toward plant products and their derivatives as an alternative to chemotherapeutants (Reverter et al., 2017). Plant products and their derivatives contain polyphenols and bioflavonoids which have anti-bacterial, anti-parasitic and immunostimulating properties that can be potentially beneficial in aquaculture (Reverter et al., 2014).

Clove oil and its derivative AQUI-S® are examples of plant products used extensively in aquaculture (Adams, 2011). These plant products are commonly used as fish anaesthetics as they have good efficacy at low concentrations (Iversen et al., 2003). In AGD research, fish are routinely sedated with clove oil for macroscopic gill observations (Adams et al., 2012) and husbandry tasks. Eugenol (4-allyl-2-methoxyphenol) is the active component of clove oil that is associated with its anaesthetic effect (Briozzo et al., 1989). Studies have shown eugenol have anti-bacterial properties (Kalemba and Kunicka, 2003; Rhayour et al., 2003; Walsh et al., 2003; Burt, 2004; Devi et al., 2010), anti-fungal properties (Kalemba and Kunicka, 2003; Pinto et al., 2009), anti-parasitic properties (Al-Yaqout and Azad, 2010) and anti-helminthic properties (Ueda-Nakamura et al., 2006). Clove oil has been widely adapted for use in aquaculture as it is inexpensive (Ross et al., 2008) and is classified as a substance that is generally regarded as safe (GRAS) by the FDA (Anderson et al., 1997).

Despite its wide usage on salmon farms and in AGD experiments, the anti-parasitic effects of clove oil and AQUI-S® against *N. perurans* parasites *in vitro* was not established. A previous study had demonstrated that freshwater bathing with AQUI-S® had no effect on AGD redevelopment in affected fish (Adams, 2011), no *in vitro* viability assessment was conducted on the amoebae. Thus, with the exception of garlic, *Allium sativum*, no other plant products have been tested against *N. perurans in vitro* (Peyghan et al., 2008; Adams, 2011).

1.4.1 Green tea extract in aquaculture

Green tea, the dried leaf and bud of *Camellia sinensis*, contains a wide array of biologically active compounds such as polyphenols, methylxanthines, essential oils, proteins, vitamins, and amino acids (Yamamoto et al., 1997). Green tea supplemented feed (100 mg kg^{-1}) was beneficial to rainbow trout *Oncorhynchus mykiss* by increasing non-specific immune function (Sheikhzadeh et al., 2011), improving fecundity and egg quality (Asadpour et al., 2012). Furthermore, green tea extract (GTE) was efficacious at removing freshwater ectoparasitic *Ichthyobodo necator* from chum salmon *Oncorhynchus keta* and masu salmon *Oncorhynchus masou* (Suzuki et al., 2006).

In vitro studies have also indicated toxicity of GTE to marine parasites affecting non-salmonid fish species such as *Cryptocaryon irritans* (Picon-Camacho et al., 2011) and *Philasterides dicentrarchi* (Leiro et al., 2004). Green tea was beneficial to kelp grouper *Epinephelus bruneus* by increasing non-specific cellular and humoral immune responses and enhancing disease resistance (Harikrishnan et al., 2011b). It also increased growth performance and disease resistance in Nile Tilapia *Oreochromis niloticus* (Abdel-Tawwab et

al., 2010). Specific effects of GTE on *O. mykiss* and other aquaculture species are summarised in Table 4.

Table 1.4. *In vivo* effects of green tea, *Camellia sinensis*

Species	Dosage	Duration of treatment	Effects on fish health	Reference
Oncorhynchus mykiss	20 mg/kg feed	30 days	Enhanced immunity	Sheikhzadeh et al. (2011)
Oncorhynchus mykiss	100 mg/kg feed	30 days	Improved oocyte quality	Asadpour et al. (2012)
Epinephelus bruneus	1000 mg/kg feed 100 mg/kg feed	28 days	Enhanced immunity	Harikrishnan et al. (2011b)
Oreochromis niloticus (L.)	500 mg/kg feed	94 days	Enhanced survival Increased growth	Abdel-Tawwab et al. (2010)
Oncorhynchus keta *Oncorhynchus masu*	0.3 % bath	30 min	Removal of external parasite	Suzuki et al. (2006)

1.4.2 Olive leaf extract in aquaculture

Olive leaf extract, *Olea europaea*, (OLE) has been used by people native to the Mediterranean basin to treat fever and diseases such as malaria (Altıok et al., 2008). The main active component of OLE are polyphenols such as oleuropein, rutin and verbacoside (Savournin et al., 2001). Olive leaf polyphenols have been studied extensively for their antioxidant properties (Papadopoulos and Boskou, 1991; De Lucia et al., 2006; Soni et al., 2006a; Di Benedetto et al., 2007) and effects on human health (Visioli and Galli, 1998; Yvonne et al., 2004; D'angelo et al., 2005b; Singh et al., 2008). OLE was observed to have *in vitro* antimicrobial properties against pathogens such as *Listeria monocytogenes, Staphylococcus aureus* (Techathuvanan et al., 2014), viral haemorrhagic septicaemia rhabdovirus (Micol et al., 2005) and *Acanthamoeba* spp. (Sifaoui et al., 2013).

In aquaculture, diets supplemented with antioxidant prevent quality degradation associated with lipid oxidation (Secci and Parisi, 2016). Studies have shown that olive leaf and olive oil by-products had beneficial effects when incorporated into fish diets at various concentrations (Table 5). When olive leaf was incorporated into diets of *Pagrus major* and *Seriola quinqueradiata*, fish had improved muscle texture (Arsyad et al., 2018) and reduced dark muscle discolouration (Oyama et al., 2010). Olive oil by-product supplemented diets improved shelf life and antioxidant activity of fish fillets when fed to *O. mykiss* (Sicuro et al., 2010b) and *Sparus aurata* (Sicuro et al., 2010a).

Table 1.5. *In vivo* effects of diets containing olive leaf and olive oil by-products

Species	Product	Dose (% of diet)	Feeding duration (days)	Effects	Reference
Pagrus major	Olive leaf powder	8	40	Increased rigidity of endomysium structure; improved muscle texture	Arsyad et al. (2018)
Seriola quinqueradiata	Olive leaf powder	1.7	42	Reduced dark muscle discolouration and improved flesh quality	Oyama et al. (2010)
Onchorynchus mykyss	Olive oil byproduct	1 or 5	94	Improved shelf life and antioxidant activity of fish fillets	Sicuro et al. (2010b)
Sparus aurata	Olive oil byproduct	1 or 5	157	Improved shelf life and antioxidant activity of fish fillets	Sicuro et al. (2010a)

1.5 Research objective and specific aims

The objective of this thesis was to identify plant products effective against *N. perurans*, the causative agent of AGD, using *in vitro* and *in vivo* experiments, and to identify the best method for *in vitro* screening of different compounds which could be used for treatment against AGD.

1.5.1 Aims

This study aimed to:

1. Elucidate the *in vitro* effects of clove oil and AQUI-S® on gill isolated amoebae (Chapter 2). It was hypothesised that clove oil and AQUI-S® would have insignificant *in vitro* effects on *N. perurans* trophozoites. AQUI-S® have been previously found to be ineffective at preventing AGD re-development during freshwater bathing (Adams, 2011).

2. Compare four commercially available kits for viability assays and manual counting using vital dye (Chapter 3). It was hypothesised that a commercially available viability estimation kit could be used on *N. perurans* trophozoites to estimate viability and obtain results similar to manual counting using vital dye.

3. Measure the *in vitro* effects of GTE and OLE on gill isolated amoebae (Chapter 4). Based on the results of Suzuki et al. (2006) and Sifaoui et al. (2013), it was hypothesised that both OLE and GTE would be effective against *N. perurans* trophozoites *in vitro*.

4. Determine the safety of GTE and OLE to Atlantic salmon at effective concentrations determined *in vitro* (Chapter 4). It was hypothesised that GTE and OLE would be safe to administer as a bath treatment at effective *in vitro* concentration. Previously, Suzuki et al. (2006) had demonstrated that GTE was safe as a bath treatment against *Ichthyobodo necator* infection in *O. keta* and *O. masou*. OLE was demonstrated to be safe when added as an in-feed treatment on *O. mykiss*.

5. Compare the efficacy of *in vivo* OLE and freshwater bath against AGD (Chapter 5). It was hypothesised that OLE would be efficacious against AGD *in vivo* based on positive *in vitro* results from Chapter 4.

Chapter Two

Effects of anaesthetics containing eugenol on *Neoparamoeba perurans*

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Chapter 2 . Effects of anaesthetics containing eugenol on *Neoparamoeba perurans*

2.1 Introduction

Amoebic gill disease (AGD) is caused by *Neoparamoeba perurans* which affects Atlantic salmon, *Salmo salar* L., during the marine production phase (Crosbie et al., 2012b). Research into AGD requires access to the pathogen and this is currently achieved by maintaining Atlantic salmon in an AGD infection tank from which amoebae are obtained from fish, either post-mortem or when moribund. To ensure the supply of amoeba a clonal culture of *N. perurans* (clone 4) was established on malt yeast seawater agar and Koch's postulates fulfilled soon after (Crosbie et al., 2012b). However, virulence of the clone was lost after around 3 years in culture (Bridle et al., 2015). Amoebae harvested from dead salmon are used for experimental AGD challenges, *in vitro* growth studies (Crosbie et al., 2012b), and screening of potential therapeutants against the amoeba (Howard and Carson, 1994; Powell et al., 2003; Powell and Clark, 2003; Florent et al., 2010; Adams et al., 2012; Crosbie et al., 2012b). Fish infected with *N. perurans* are routinely handled in the course of macroscopic gill observations (Adams et al., 2012) and to obtain gill swabs to determine disease status during *in vivo* experiments.

Exposure of *N. perurans* to anaesthetics occurs when fish are sedated during AGD experiments and when moribund fish are euthanised. Clove oil and AQUI-S® are examples of commonly used fish anaesthetics; both are eugenol-based and demonstrate good efficacy at low concentrations (Iversen et al., 2003). Around 90-95% of clove oil is eugenol (4-allyl-2-methoxyphenol) which is the active component (Briozzo et al., 1989). AQUI-S® was developed based on eugenol and contains 50% iso-eugenol (2-methoxy-4-propenylphenol)

and 50% polysorbate 80 (Ross and Ross, 1999). Eugenol has been attributed with anti-bacterial properties (Kalemba and Kunicka, 2003; Rhayour et al., 2003; Walsh et al., 2003; Burt, 2004; Devi et al., 2010), anti-fungal properties (Kalemba and Kunicka, 2003; Pinto et al., 2009), anti-parasitic properties (Al-Yaqout and Azad, 2010) and anti-helminthic properties (Ueda-Nakamura et al., 2006). Unlike anaesthetics such as benzocaine and MS-222 (Tricaine Methanesulfonate), clove oil is inexpensive with no withdrawal time (Ross et al., 2008) and classified to be a substance that is generally regarded as safe (GRAS) by the FDA (Anderson et al., 1997).

It was unknown if eugenol-based fish anaesthetics exert any *in vitro* anti-microbial effect on *N. perurans* despite regular usage in AGD experiments. Therefore, the aim of the present study was to determine the effects of clove oil and AQUI-S® on *N. perurans,* both those isolated from dead fish (host-associated) and clone 4 *in vitro* in terms of survival and growth post exposure and the ability to attach to a surface.

2.2 Materials and methods

Amoebae were obtained from donor fish from the experimental AGD infection tank at the University of Tasmania's aquaculture centre. All fish in this trial were approved for experimentation by the University of Tasmania, Australia (Animal Ethics Committee Permit No. A13840). Host-associated *N. perurans* trophozoites were isolated from salmon according to Morrison et al. (2004). Trophozoites of clone 4 were obtained by dislodgement from agar with a gentle stream of 0.2 µm filtered seawater (FSW) and then the cell suspension was poured onto clean empty Petri dishes, covered and allowed to adhere for 1 h at 18 °C. Plates were then washed with FSW and cells that had adhered to the plates were

dislodged with 0.05% trypsin-EDTA (Gibco). Cells were washed and concentrated by centrifugation (450 x *g* for 5 min, Eppendorf Centrifuge 5810 R).

The concentrations of clove oil tested in this experiment were based on concentrations previously used for euthanasia and anaesthesia in other experiments, those ranged between 20 µL L^{-1} and 50 µL L^{-1} (Powell et al., 2000; Fisk et al., 2002; Roberts and Powell, 2003; Leef et al., 2005b; Leef et al., 2005a; Powell et al., 2005; Jones et al., 2007; Leef et al., 2007a; Leef et al., 2007b; Powell et al., 2007; Roberts and Powell, 2008; Powell et al., 2009; Villavedra et al., 2010). The concentrations of AQUI-S® tested in this experiment were based on concentrations previously used for euthanasia and anaesthesia in other experiments, those ranged between 15 µL L^{-1} and 40 µL L^{-1} (Taylor et al., 2010; Crosbie et al., 2012a; Leef and Nowak, 2013; Maynard et al., 2016; Wright et al., 2018; English et al., 2019). The exposure time used in this experiment is based on previous fish handling events by the author. Time and concentrations of clove oil and AQUI-S® used in this experiment are described in Table 2.1 and 2.2.

2.2.1 Ability to attach to a surface post anaesthetics exposure

To determine the impact on attachment post anaesthetics exposure on both host-associated and clone 4 trophozoites, 100 amoebae in 100 µL of 0.2 µm FSW were inoculated on 96-well cell culture plates (Corning®) in triplicate wells and allowed to attach for 1 h at 18 °C. The initial 100 µL of FSW was then removed and replaced with 100 µL of 0.2 µm FSW containing the various concentrations of anaesthetics and incubated at 18 °C as shown in Table 2.1. After incubation, the 100 µL of FSW containing anaesthetics and any detached amoebae were removed and replaced with the 100 µL of 0.2 µm FSW. Amoebae that

remained attached were counted and the mean numbers of detached amoebae calculated as a percentage of the initial total. FSW only was used as control. The viability of detached cells was assessed as outlined in section 2.2.2.

Table 2.1. Anaesthetics concentration and exposure time for determination of detachment of *N. perurans* trophozoites after anaesthetics exposure.

Exposure time	Anaesthetic	Exposure concentration (µL L^{-1})
10 minutes	AQUI-S®	5, 10, 20, 40
	Clove oil	10, 20, 40, 80
	Control	0.2 µm filtered seawater
120 minutes	AQUI-S®	2.5, 5, 10, 20
	Clove oil	5, 10, 20, 40
	Control	0.2µm filtered seawater

2.2.2 Anaesthetics exposure and assessment of amoebae viability and growth

For each trial, a suspension of 1000 amoebae in 500 µL was placed into triplicate 1 mL microcentrifuge tubes and incubated with 500 µL of either clove oil (CL032 85% eugenol Chem-supply) or AQUI-S® solutions as outlined in Table 2.2. To remove the majority of the anaesthetic after the designated incubation period, the amoebae were concentrated by centrifuge at 13, 000 x *g* for 15 s, after which 900 µL of supernatant was removed and

replaced with 900 µL of 0.2 µm FSW and the suspension was mixed by vortex for 5 s, and then concentrated again by centrifuge as described above.

Table 2.2 Anaesthetics concentration and exposure time for determination of growth and viability of *N. perurans* trophozoites.

Exposure time	Anaesthetic	Exposure concentration (µL L^{-1})
20 minutes	AQUI-S®	25
	Clove oil	80
	Control	0.2µm filtered seawater
120 minutes	AQUI-S®	5, 10
	Clove oil	20, 40
	Control	0.2 µm filtered seawater

Thereafter the entire supernatant was removed, leaving behind the pellet which was re-suspended in 100 µL of 0.2 µm FSW. The viability of amoebae was then assessed by the inclusion of a vital dye (neutral red; Sigma). A 20 µL aliquot of the amoeba suspension was added to 20 µL of neutral red solution (50 µg mL^{-1} in phosphate buffered saline) in a 1 mL microcentrifuge tube and incubated at room temperature for 20 min. Excess dye was then removed by adding 900 µL of 0.2 µm FSW, mixing by vortex for 10 s and centrifugation at 13,000 x *g* after which 900 µL of supernatant was removed, leaving behind the amoebae.

Cells were examined using a haemocytometer and 10 µL of cell suspension and those that had taken up the dye into vacuoles were considered viable. Percentage viability was calculated by dividing total viable count by total number of cells. Viability of amoebae was expressed as percentage viable amoebae.

To determine growth, 15 amoebae based on volume of amoebae suspended in FSW from each treatment were inoculated into individual wells of a 96-well cell culture plate (Corning®). Viability of amoebae was determined as described above. Each well was filled with 100 µL of malt yeast agar (0.01% malt, 0.01% yeast, 2% Bacto Agar, 0.2 µm FSW at 35‰ salinity, 18 °C) in triplicate. Each well was then overlayed with 100 µL of 0.2 µm FSW. Total amoebae count in each well were performed every 24 h for 4 days. Counts were performed using an inverted microscope at 40 x magnification; a grid pattern was marked onto bottoms of individual wells to aid counting. All the amoebae in each well were counted but time used to perform counts was not standardised between individual wells. Growth was expressed as the mean number of amoebae in each treatment.

2.2.3 Statistical Analysis

One-way ANOVA (SPSS® Version 20; IBM®) was used to determine the effect of treatment on amoeba viability, growth and attachment. Data were tested for normality and homogeneity using Shapiro–Wilk test and Levene's test respectively. Data were subjected to transformation if normality was not met and the Welch test was used when homogeneity was not met. Tukey's HSD was used for comparisons of means where assumptions of normality and homogeneity were met. $P < 0.05$ was adopted for the rejection of the null hypothesis.

2.3 Results

Attachment of both host-associated and clone 4 trophozoites was affected when exposed to clove oil at 80 µL L^{-1} for 10 min but not AQUI-S (Figure 2.1). Significantly more amoebae had detached after exposure to clove oil at 80 µL L^{-1} for 10 min compared to control (host-associated, $F = 8.321$, df 8,18, $P < 0.001$, Clone 4, $F = 4.745$, df 8,18, $P = 0.003$). The mean percentage of amoebae detached after exposure to clove oil at 80 µL L^{-1} for 10 min was 9% for the host-associated and 16% for clone 4 compared to the respective control averages of 3% and 7% amoebae detached (Figure 2.1).

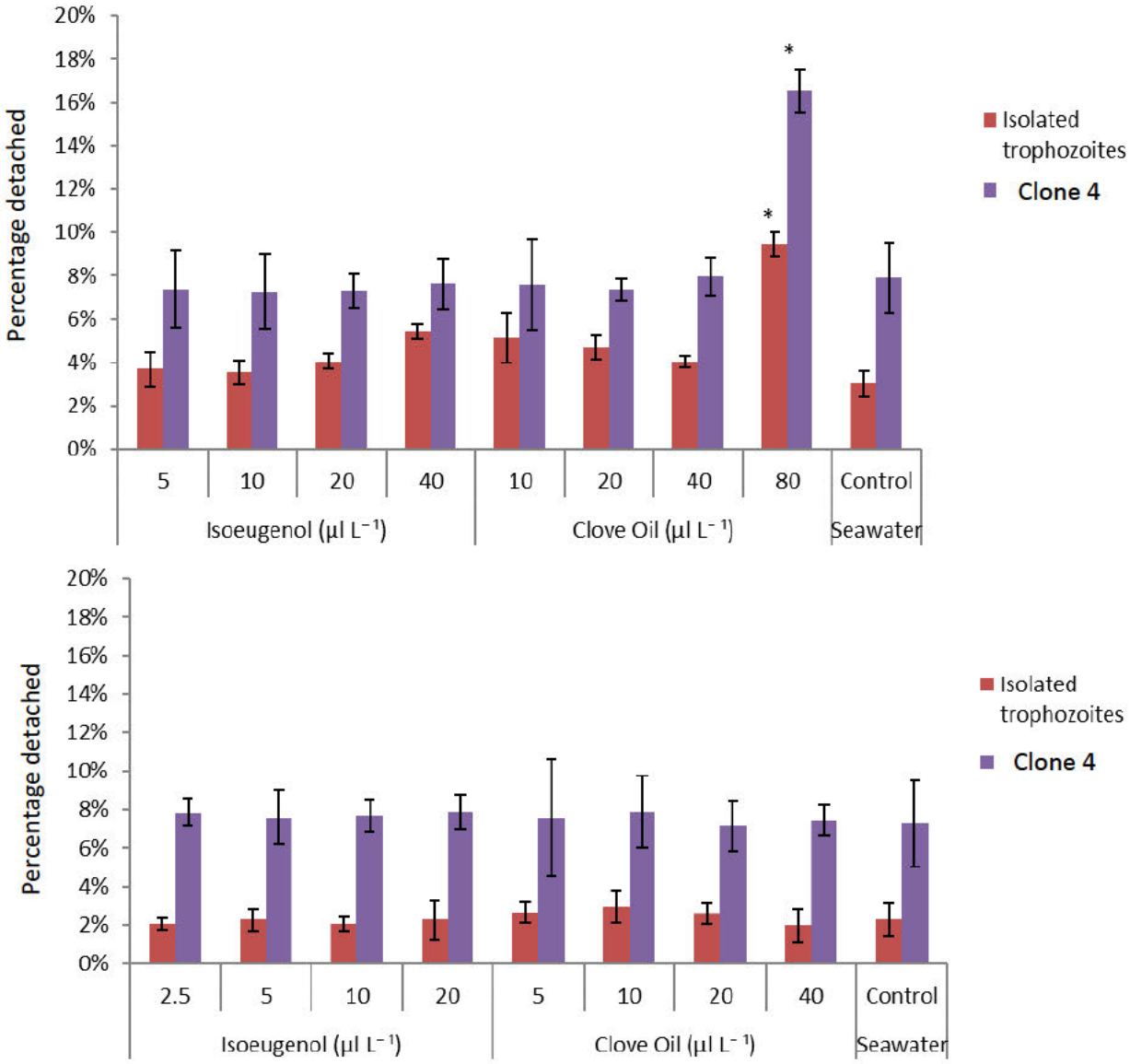

Figure 2.1 Percentage of isolated trophozoites and Clone 4 trophozoites that detached after anaesthetic exposure for 10 min (top graph) and 120 min (bottom graph). Asterisk indicates significant difference between treatments ($P < 0.05$). Results are presented as mean percentage of amoebae that had detached after anaesthetics exposure ± standard error.

Exposure to clove oil at 80 µL L^{-1} for 10 min resulted in increased detachment of previously attached trophozoites. There was no effect on the viability of all the detached amoebae as

assessed by neutral red vital dye. No increase in detachment was observed in amoebae that were exposed to anaesthetics at for 10 min at 40 µL L^{-1} or lower concentrations (Figure 2.1). Attachment of host-associated and clone 4 trophozoites was neither affected by clove oil nor AQUI-S when exposed to concentrations of 40 µL L^{-1} or lower for 120 min (Figure 2.1). There was no significant difference in the numbers of detached amoebae between the treatment cases and the control (fresh isolate, F = 0.244, df 8, 18, P = 0.976, Clone 4, F = 0.026, df 8, 18, P = 0.99999). The mean percentage of amoebae detached after exposure for 120 min to clove oil and AQUI-S at between 5 to 40 µL L^{-1} was 2%.

There were no significant effects of anaesthetic exposure on either host-associated or clone 4 in terms of growth (Figure 2.2, host-associated; F = 0.108, df 7, 88, P = 0.998, clone 4, F = 0.035, df 7, 88, P = 0.99998) and viability (host-associated, 20 min treatment F = 2.626, df 2, 6, P = 0.152, 120 min treatment F = 2.297, df 4, 10, P = 0.131, clone 4, 20 min treatment F = 0.18, df 2, 15, P = 0.837, 120 min treatment F = 0.626, df 4, 25, P = 0.648).

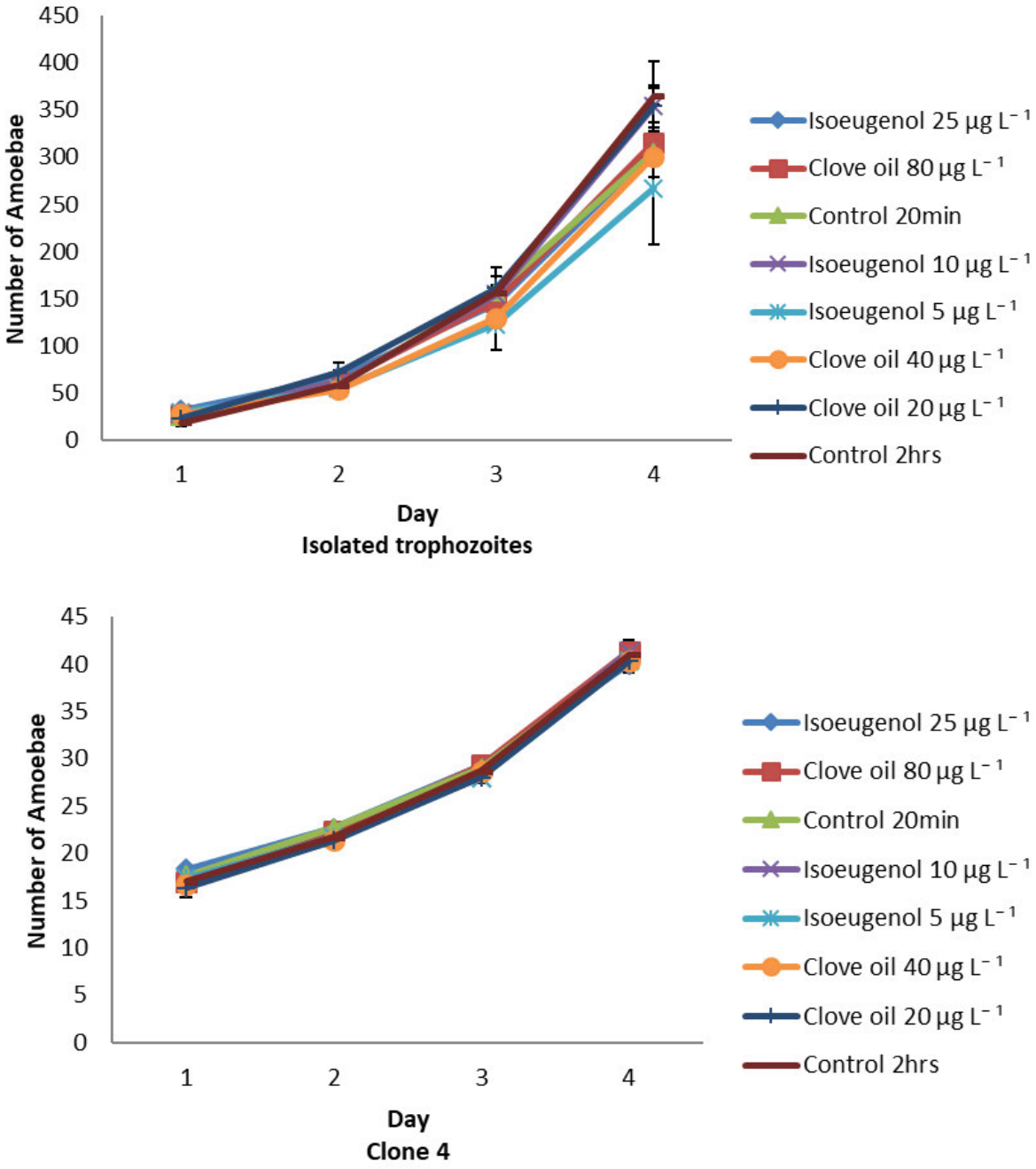

Figure 2.2 Growth of isolated and clone 4 amoebae over 4 days after exposure to select concentration of anaesthetics in Table 2.1. Results are presented as mean number of amoebae in well ± standard error.

2.4 Discussion

Exposure to clove oil at 80 µL L^{-1} for 10 min caused a significant increase in the number of detached cells of both the host-associated and clone 4 compared to the lower concentrations. Similar to results from this study, clove oil affected attachment (IC_{50} of 0.134 µL L^{-1}) of *Giardia lamblia*, a protozoan parasite, onto cell culture vials (Machado et al., 2011). Gross signs due to attachment of *N. perurans* on host gill epithelium are used to confirm the gross presence of AGD on anaesthetised fish during *in vivo* experiments (Adams et al., 2004; Adams et al., 2012). Attachment of *N. perurans* trophozoites to host gill epithelium is also critical for disease progression during experiments (Powell et al., 2008). While an increase in detachment of trophozoites was observed, this observation was made at twice the concentration of clove oil normally used. However it is unknown if trophozoite attachment onto host gill epithelium would be affected as attachment is likely to be dependent on attachment proteins (Garate et al., 2005). Even though trophozoite attachment was affected by high concentration of clove oil, it is unlikely to affect future *in vivo* AGD experiments as the effect was only observed at 80 µL L^{-1}. Despite the results being statistically significant, over 90% of amoebae in the experiment remained attached and all detached amoebae were viable. Based on the current results, AQUI-S® is a more suitable anaesthetic for AGD experiments because there were no negative effects across a range of concentrations after a single exposure.

When *N. perurans* trophozoites were subjected to repeated 20 min exposure to 17 mg L^{-1} AQUI-S® over 28 days, an increased proportion of amoeba remained attached to their substrate as compared to control (Chance et al., 2017). This might have led to an artificially

lengthen duration of attachment during *in vivo* experiment when fish are sedated regularly with AQUI-S®. This might have resulted in *N. perurans* trophozoites having more time to colonise the gill substrate (Wiik-Nielsen et al., 2016) leading to accelerated disease progression which may not be an accurate representation of disease progression found under field conditions.

Clove oil at concentrations of 10-80 µL L^{-1} and AQUI-S® at concentrations of 5-40 µL L^{-1} had no effect on the growth and viability of *N. perurans* either host-associated or clone 4 trophozoites *in vitro*. The use of anaesthetics in research facilitates handling fish during gill observations, ensures humane euthanasia of moribund fish from which *N. perurans* are harvested and for destroying fish at the conclusion of experiments. While plant extracts rich in eugenol demonstrated growth inhibition of protozoan parasites such as *Leishmania amazonensis*, *L. chagasi* (Braga et al., 2007) and *Trypanosoma cruzi*, (Santoro et al., 2007) growth inhibitions were only observed after 24 to 72 h of exposure and at concentrations higher than those used for this study.

The results of this experiment indicate that exposure to clove oil or AQUI-S® during the harvest of *N. perurans* from salmon is unlikely to have an impact on the viability of the amoebae to be used in subsequent experiments because exposure times and concentrations tested were higher than those used when handling or anaesthetising fish. Therefore, harvesting of *N. perurans* trophozoites from AGD-affected fish euthanised by clove oil and AQUI-S® at these concentrations is unlikely to affect subsequent viability, growth and attachment of the amoebae. Anaesthetising AGD-affected fish in at these concentrations is also unlikely to hinder experimental disease induction and progression.

Chapter Three

Assessment of *in vitro* viability assays for

Neoparamoeba perurans

Chapter 3 Assessment of *in vitro* viability assays for *Neoparamoeba perurans*

3.1 Introduction

In vitro viability assessment of *Neoparamoeba perurans* is a useful tool to identify potential treatment agents and provides data for subsequent *in vivo* studies. The most frequently used method is manual counting using a haemocytometer after uptake of vital stain Neutral Red (Adams et al., 2012; Chance et al., 2017). Other previously used methods include manual counting using a haemocytometer after staining with trypan blue for dye exclusion (Powell et al., 2003; Powell and Clark, 2003; Florent et al., 2010), minimum inhibitory concentration using agar growth media (Howard and Carson, 1993; Howard, 2001) and ability of amoebae to attach onto a plastic substrate (Wright et al., 2018). However, trypan blue exclusion was not suitable for *N. perurans* cells that had been through a freeze/thaw cycle as it only indicated cell membrane integrity but not cell viability (Nowak et al., 2004). Neutral red inclusion assay is preferred because cell viability is indicated by cellular metabolic activity (Nowak et al., 2011).

Microplate assays were developed as techniques to assess the *in vitro* effects of drugs and other compounds on cell lines, bacteria and viruses (Vega-Avila and Pugsley, 2011). These assays have the advantage of being able to use only minimal amount of media and reagents, furthermore it is easily automated which increases the screening throughput (Vega-Avila and Pugsley, 2011). The viability assays chosen for this study are all commercially available, high throughput cell viability assay using different markers as indicators of viability. Four assays were compared in this study, CellTiter-Fluor™, CytoTox-ONE™, CellTiter-Glo® and CellTiter-Blue®. Of the four viability assays chosen, only CellTiter-Blue® have been used on

N. perurans for commercial testing (Nowak, pers. comm.). The other three viability assays have not been used previously on *N. perurans*. The viability reagents CellTiter-Fluor™ and CytoTox-ONE™ measure cell membrane integrity as an indicator of viability (Table 3.1). CellTiter-Fluor™ reagent contains cell permeable substrates that enter cells where they are cleaved by live-cell protease activity to produce a fluorescence signal. This protease activity is conserved and constitutive within live cells thus serves as a cell viability marker (Niles et al., 2007). CytoTox-ONE™ measures lactate dehydrogenase (LDH) that is released from cells with compromised cell membranes. LDH is measured by providing lactate, NAD+ and resazurin substrates together with diaphorase. This is a well-established method for the determination of non-viable cells with results comparable to radioisotope release assays (Korzeniewski and Callewaert, 1983; Decker and Lohmann-Matthes, 1988). CellTiter-Glo® (Table 3.1) measures the amount of ATP, an indicator of metabolically active cells, which is directly proportional to the number of viable cells (Crouch et al., 1993). CellTiter-Blue® (Table 3.1) measures viability based on the ability of viable cells to reduce resazurin into resorufin (O'brien et al., 2000). Viability results generated using resazurin were demonstrated to be similar to results obtained by manual counting using Congo red exclusion dye (Heredero-Bermejo et al., 2013).

Table 3.1 Comparison between viability assays

Viability assay	Viability assumption	Mode of action	Reference
CellTiter-Fluor™	Live cells have intact cell membranes	Reagent contains cell permeable substrates that enter cells where they are cleaved by live-cell protease activity is conserved and constitutive within live cells thus serves as a cell viability marker	Niles et al. (2007)
CytoTox-ONE™	Cells with damaged membranes are non-viable	Lactate dehydrogenase is released from cells with damaged cell membranes. LDH is measured by providing lactate, NAD+ and resazurin substrates together with diaphorase	Korzeniewski and Callewaert (1983) Decker and Lohmann-Matthes (1988)

CellTiter-Glo®	Viable cells are metabolically active cells	Measures the amount of ATP, which is directly proportional to the number of viable cells	Crouch et al. (1993)
CellTiter-Blue®	Viable cells are metabolically active cells	Viable cells are able to reduce resazurin into resorufin	O'brien et al. (2000)

Previous studies have successfully used alamarBlue, same active ingredient as CellTiter-Blue®, to investigated the efficacy of olive leaf extract against *Acanthamoeba castellanii* Neff (Sifaoui et al., 2013) and efficacy of alkylphosphocholines against *A. castellanii* and *Acanthamoeba polyphaga* (McBride et al., 2005). Using alamarBlue McBride et al. (2005) developed a microplate assay that is suitable for automation and high throughput screening. However, it is important to note that the suitability of viability assay may be strain or species dependent, despite being successfully used with protozoa, viability assay should be optimised for individual strain or species (Heredero-Bermejo et al., 2013).

The recent interest in using plant products instead of chemotherapeutants has led to the identification of potentially antimicrobial or antiparasitic plant products (Reverter et al., 2014). Plant products such as ginger *Zingiber officinale* (Levy et al., 2015) and black pepper *Piper nigrum* (Kumar et al., 2012a) have *in vivo* antimicrobial properties (Table 3.2). Despite knowledge of plant products with antimicrobial effects, only garlic *Allium sativum* (Peyghan et al., 2008) and eugenol (Chapter 2) has been tested against *N. perurans*.

Table 3.2 List of plant products with either *in vitro* or *in vivo* antimicrobial activity.

Plant product (scientific name)	Species	Activity	Effective concentration	Reference
Ginger *Zingiber officinale*	*Gyrodactylus turnbulli*	*In vivo*	5 mg L^{-1}	Levy et al. (2015)
Cinnamon *Cinnamomum zylancium*	*Staphylococcus aureus*	*In vitro*	4% (w/v)	Ağaoğlu et al. (2007)
Cumin *Cuminum cyminum* L	*Staphylococcus aureus*	*In vitro*	4% (w/v)	Ağaoğlu et al. (2007)
Onion *Allium cepa*	*Escherichia coli*	*In vitro*	1 mg mL^{-1}	Rauha et al. (2000)
Turmeric *Curcuma longa*	*Vibrio parahaemolyticus*	*In vitro*	5% (w/v)	Yano et al. (2006)
Chilli *Capsicum frutescens*	*Listeria monocytogenes*	*In vitro*	1% (w/v)	Leuschner and Ielsch (2003)
Coriander *Coriandrum sativum* L.	*L. monocytogenes* *S. aureus* *Escherichia coli*	*In vitro*	0.02% (v/v)	Delaquis et al. (2002)

Black pepper *Piper nigrum*	*Argulus spp*	*In vivo*	9 mg L^{-1}	Kumar et al. (2012a)
Olive leaf extract *Olea europaea*	*Acanthamoeba*	*In vitro*	33 µg mL^{-1}	Sifaoui et al. (2013)
Green tea extract *Camellia sinensis*	*Ichthyobodo necator*	*In vivo*	0.03% (w/v)	Suzuki et al. (2006)

In the present work, the viability assays were evaluated using a stock solution made up of viable and non-viable cells. Manual counting using two different dyes was compared against the four viability assays cited above to determine their suitability as alternative viability assessment methods for high throughput screening of potential anti-*N. perurans* compounds. Using the most suitable viability assay, plant products (Table 3.2) were screened for *in vitro* effects against *N. perurans*.

3.2 Materials and methods

Amoebae were isolated using a previously established method (Morrison et al., 2004). Donor fish (dead) were obtained from a dedicated AGD infection tank located at the University of Tasmania Aquaculture centre Newhnam campus. Gills were excised, dissected into individual gill arches and placed into 50 mL tubes filled with 0.2 µm filtered seawater. The tube was agitated for 1 min to dislodge amoebae from the gills. Solution containing suspended amoebae was poured into petri dishes and the amoebae were allowed to attach at room temperature for 1 h. Plates were rinsed with 0.2 µm filtered seawater and amoebae

were dislodged using 0.05% trypsin-EDTA. Dislodged cells suspended in trypsin were washed in 0.2 µm filtered seawater and concentrated by centrifugation (450 *g* for 5 min, Eppendorf Centrifuge 5810 R).

3.2.1 Preparation of non-viable amoebae

To obtain non-viable amoebae (heat inactivated) with compromised cell membrane (Ebrahimi et al., 2018), amoebae were suspended in 0.2 µm filtered seawater and incubated at 60 °C for 1 h. After incubation, amoebae were washed with 0.2 µm filtered seawater. To obtain non-viable amoebae (formalin inactivated) with intact cell membrane (Fox et al., 1985), 100 µL of 10 % formalin was added to 9.9 mL of amoebae suspension and incubated at 18 °C for 30 min. Cells were washed twice by centrifuging at 13,000 *g* for 15 s and removing supernatant after adding 9 mL of 0.2 µm filtered seawater. After both formalin and heat treatment of amoebae, viability was verified using methods described in the following section.

3.2.2 Manual counting using vital dyes

To perform manual counting using trypan blue vital dye, 10 µl of 1 % trypan blue solution was added to 10 µl of amoebae suspension, this mixture was loaded immediately into a haemocytometer and examined under a microscope at 100x magnification. Cells that excluded the dye were considered as viable while cells that stained blue were considered as non-viable.

To perform manual counting using neutral red vital dye, 20 µL of Neutral Red solution was added to 20 µL of amoebae suspension and incubated at 18 °C for 20 min. Excess dye was

removed by adding 800 µL of 0.2 µm filtered seawater and centrifuged at 13,000 *g* for 15 s (Eppendorf 5415D). 800 µL of supernatant containing the excess dye was removed. After three washes, the washed amoebae suspension was loaded into a haemocytometer and examined under microscope at 100x magnification. Cells that did not retain the dye were considered as non-viable and cells that had internalized the dye were considered as viable. Viability of amoebae used for optimisation of all assays and preparation of stock solution was validated using vital dyes cited above. Each method had 3 replicates and counts were performed in quintuplicate for each replicate. Results are presented as percentage viability ± standard error.

$$\frac{number\ of\ viable\ cells}{total\ number\ of\ cells} * 100 = \%\ viability$$

3.2.3 Optimisation of the four viability assays

The four viability assays used were: CellTiter-Fluor™, CytoTox-ONE™, CellTiter-Glo® and CellTiter-Blue®; all were purchased from Promega Australia. Serial 1:1 dilution of viable cells was used to generate standard curves for all four assays according to manufacturer instructions. Two sets of serial dilutions were performed for each viability assay. One set starting with 1,500 cells in 100 µL, while another starting with 10,000 cells in 100 µL. Each set of serial dilutions had 3 replicates. Plates were read using Tecan© Infinite 200. Signals generated by viability assays were expressed as either relative fluorescence units (RFU) or relative luminescence units (RLU).

3.2.4 Stock solution

Stock solution made up of 50% live amoebae and 50% dead amoebae was prepared by mixing viable and inactivated amoebae in the following ratio: 50 % viable, 25 % heat inactivated and 25 % formalin inactivated. This stock solution was then used to evaluate both manual counting and viability assays for accuracy.

3.2.5 In vitro effects of plant products on N. perurans trophozoites

Chilli, coriander, turmeric, black pepper, ginger, onion, cumin and cinnamon (unknown manufacturer, sold as Coles Home Brand) were prepared as a 0.1 % solution. 0.1 g of plant product was added to 100 mL of 0.2 µm filtered seawater, incubated for 1 h at 18 °C and filtered with a 0.2 µm filter. Green tea extract (GTE, Bulk Nutrients, EGCg 40 %) and olive leaf extract (OLE, Nature's Care Manufacture Pty. Ltd., 1.7 % oleuropein) were prepared similarly but at 0.015 and 0.0075 mg L^{-1} GTE; 0.078 and 0.039 mg L^{-1} OLE. 100 µL of amoebae (n = 1000) were added to wells of a 96 well plate. The plate was incubated at 18 °C for 1 h to allow amoebae to adhere to the wells. Seawater was removed and replaced with 100 µL of plant extracts and incubated for 2 h. Viability was determined using CellTiter-Glo® according to manufacturer instructions. All plant extracts were tested in triplicates. 0.2 µm filtered seawater was used as negative control and freshwater was used as the positive control. Results are presented as % viability ± standard error as determined by viability assay.

3.2.6 Statistical Analysis

Regression analysis (SPSS® Version 20; IBM®) was used to determine the relationship between signal generated by the viability assays and the number of live *N. perurans* trophozoites. One-way ANOVA (SPSS® Version 20; IBM®) was used to determine the effect of vital stains on viability estimation and the effect of different plant products on the viability of *N. perurans* trophozoites *in vitro*. Data were tested for normality and homogeneity using Shapiro–Wilk test and Levene's test respectively. Data were subjected to transformation if normality was not met and the Welch test was used when homogeneity was not met. Tukey's HSD was used for comparisons of means where assumptions of normality and homogeneity were met. $P < 0.05$ was adopted for the rejection of the null hypothesis.

3.3. Results

There was a significant regression equation in all assays between the signal generated and the number of viable cells when cells were 1:1 serially diluted stating from 1,500 viable cells (Table 3.3, Figure 3.1).

Table 3.3 Regression analysis of signal generated by viability assay and viable *N. perurans* trophozoites when trophozoites were 1:1 serially diluted stating from 1,500 viable trophozoites.

Viability assay	F	*P*	R^2
CellTiter-Blue®	F (1,13) = 418.89	$P < 0.001$	0.97
CytoTox-ONE™	F (1,13) = 141.287	$P < 0.001$	0.91

CellTiter-Glo®	F (1,13) = 1955.56	*P* < 0.001	0.99
CellTiter-Fluor™	F (1,13) = 806.97	*P* < 0.001	0.98

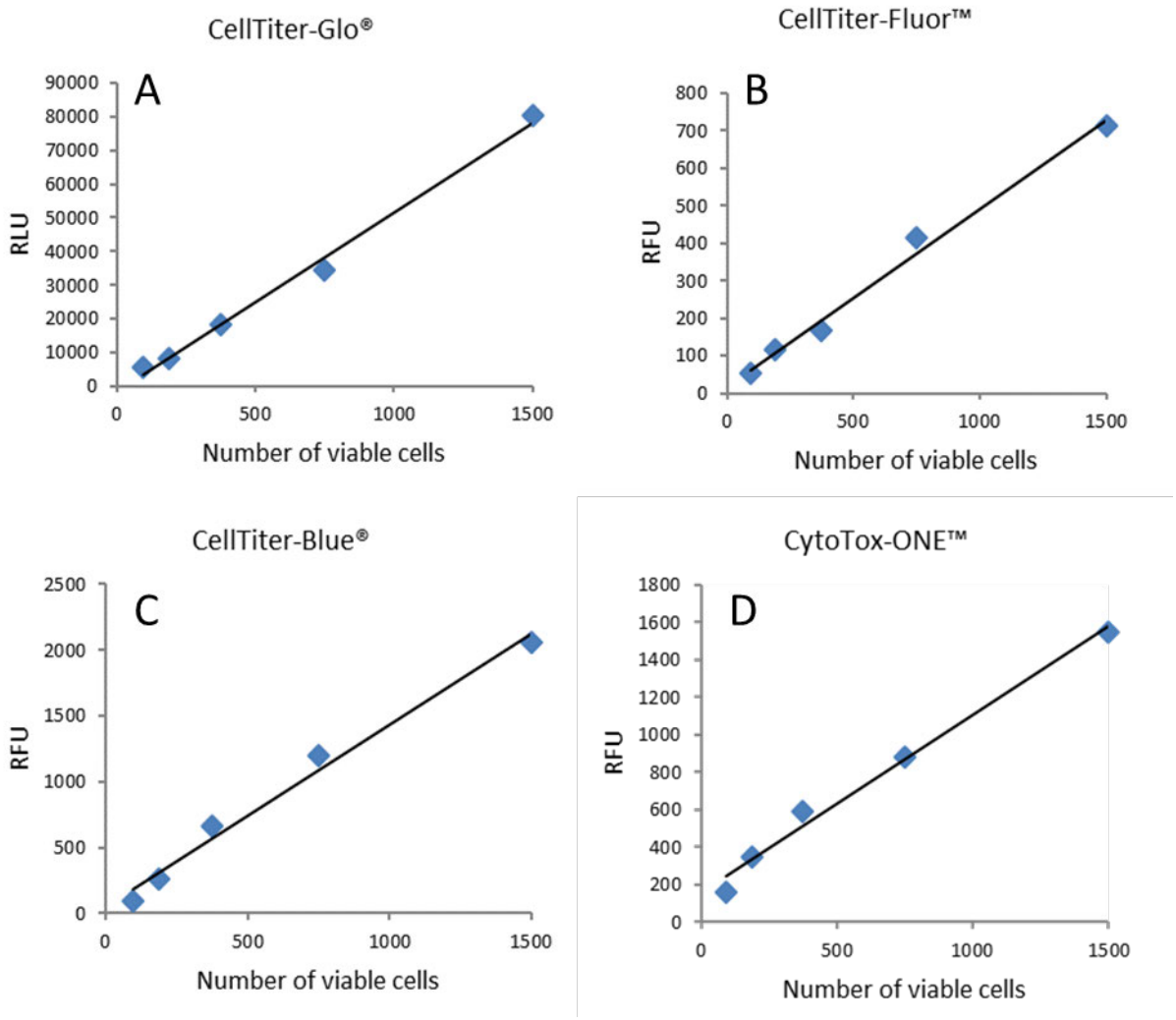

Figure 3.1. Luminescence (A) and fluorescence (B-D) signals produced by CellTiter-Blue®, CellTiter-Glo®, CellTiter-Fluor™ and CytoTox-ONE™. Results shown for linear relationship between number of viable cells and signal generated. Maximum number of viable cells was 1500.

The number of viable cells were not significantly associated with signal generated by CellTiter-Blue® and CytoTox-ONE™ when cells were 1:1 serially diluted starting from 10,000 viable cells (Table 3.3). The number of viable cells were significantly associated with signal generated by CellTiter-Glo® and CellTiter-Fluor™ when cells were 1:1 serially diluted starting from 10,000 live cells (Table 3.4, Figure 3.2).

Table 3.4 Regression analysis of signal generated by viability assay and viable *N. perurans* trophozoites when trophozoites were 1:1 serially diluted stating from 10,000 viable trophozoites.

Viability assay	F	*P*	R^2
CellTiter-Blue®	F (1,13) = 0.256	*P* > 0.05	0.01
CytoTox-ONE™	F (1,13) = 0.955	*P* > 0.05	0.06
CellTiter-Glo®	F (1,13) = 2971.15	*P* < 0.001	0.99
CellTiter-Fluor™	F (1,13) = 41.75	*P* < 0.001	0.76

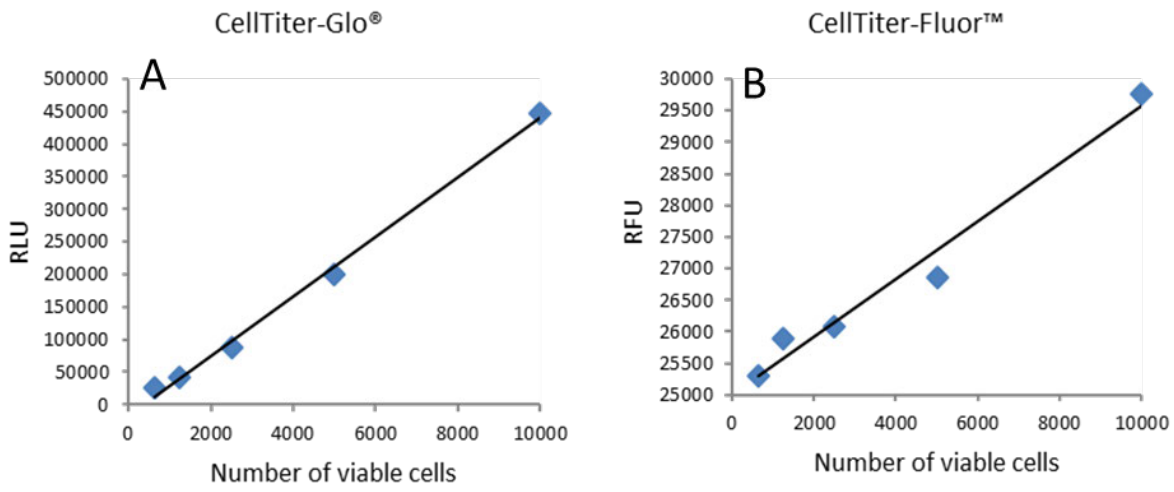

Figure 3.2. Luminescence (A) and fluorescence (B) signals produced by CellTiter-Glo® and CellTiter-Fluor™. Results shown for linear relationship between number of viable cells and signal generated. Maximum number of viable cells was 10000.

Signals generated by viability assays were independent of methods of amoebae inactivation. However, when manual counting was performed using trypan blue, viability was significantly greater (Table 3.4). Viability of the stock solution assessed using trypan blue was significantly higher ($P < 0.05$) as compared to neutral red and viability assay. When neutral red was assessed against viability assays, the viability of stock solution was not significantly different. Regression analysis indicated that CellTiter-Glo® had the highest R^2, thus it was chosen to evaluate *in vitro* effects of plant products against *N. perurans* trophozoites (Table 3.5).

Table 3.3. Viability test result of stock solution. Results are presented as mean ± standard error. Cells that internalised Neutral Red inclusion dye were classified as

viable, while cells that excluded Trypan blue exclusion dye were classified as viable. Asterisk indicates significant difference ($P < 0.05$).

Viability method	% Viability
Neutral red inclusion dye	48.4% ± 2.1
Trypan Blue exclusion dye	71.3% ± 1.1*
CellTiter-Blue®	54.5% ± 3.4
CellTiter-Glo®	50.3% ± 0.6
CellTiter-Fluor™	53.3% ± 0.7
CytoTox-ONE™	52.3% ± 1.7

Table 3.4. Viability of *N. perurans* after exposure to plant products. Viability estimated using CellTiter-Glo®. Results are presented as mean ± standard error. Asterisk indicates significant difference.

Plant product (% v/w)	% Viability
Chili (0.1%)	84.9% ± 1.9*
Coriander (0.1%)	91.1% ± 0.7
Turmeric (0.1%)	92.8% ± 0.7
Black pepper (0.1%)	77.8% ± 2.1*
Ginger (0.1%)	92.7% ± 0.9
Onion (0.1%)	92.1% ± 1.2
Cumin (0.1%)	92.3% ± 1.7
Cinnamon (0.1%)	92.7% ± 1.4

GTE (0.015%)	0.2% ± 0.1*
GTE (0.0075%)	19.0% ± 0.4*
OLE (0.078%)	0.3% ± 0.1*
OLE (0.039%)	21.1% ± 0.3*
Positive control	98.01% ± 0.14
Negative control	0%

3.4. Discussion

The assays used in this study achieved accuracy similar to manual counting (neutral red). When viability assays were compared against manual counting using cells in stock solution, trypan blue vital dye showed significantly higher ($P < 0.001$) results. During formalin inactivation of viable trophozoites, the cell membrane was fixed thus leading to false positives with trypan blue. As a result of the false positive results, neutral red is preferred over trypan blue (Nowak et al., 2004). Cell membrane integrity had no effect on the accuracy of all the viability assays tested and neutral red vital dye. Despite known interference associated with autofluorescence of formaldehyde-fixed cells (Del et al., 1989; Baschong et al., 2001; Davis et al., 2014), negative controls indicated that the assays used in this study were unaffected.

Of the four viability assays tested, CellTiter-Glo® had several advantages over the other three assays. (1) CellTiter-Glo® is an “endpoint addition” reagent, unlikely to affect results as only screening compounds and trophozoites are in contact throughout the assay. (2) The probability of operator-related errors is reduced as the assay is based on the addition of a single reagent. (3) The assay measures ATP, which is a natural cellular product that is

produced during cell metabolism. The level of ATP is closely regulated in healthy cells that are metabolically active, which makes ATP an ideal biomarker (Lundin et al., 1986; Crouch et al., 1993). There is no additional strain on cells to reduce or oxidise an introduced substance to generate a signal. (4) It is rapid and only requires 10 mins of incubation to quantify viable cells. (5) After reagent is added, the signal generated is stable and has a half-life of five hours (Hannah et al., 2001; Riss et al., 2005). This enables batch processing of plates and reduces inter-plate signal variations. (6) ATP based assay is highly sensitive and able to detect as few as 10 cells (Hannah et al., 2001). (7) The assay also has a high signal to background ratio which allows miniaturisation into 1536 well formats (Borawski et al., 2007; Severson et al., 2007).

Despite several advantages, CellTiter-Glo® is susceptible to temperature flux; equilibration to constant room temperature is required to minimise thermal gradients created by plate well position (Hannah et al., 2001). Like other metabolic chemistry-based assays, ATP is subjected to positive or negative modulation by test reagent independent of loss of viability resulting in inaccurate data (Sánchez-Alcázar et al., 1997; Shchepina et al., 2002). Lastly, all commercial formulations of ATP based assays are susceptible, to a certain degree, to luciferase inhibition of various degrees by small molecule compounds (Hannah et al., 2001; Kashem et al., 2007). However, improvements in the formulation of luciferase reagent may minimise the degree of this inhibition (Hannah et al., 2001; Kashem et al., 2007). Overall CellTiter-Glo® is the most suitable viability assay for use with *N. perurans*.

Further tests of plant products using CellTiter-Glo® viability assay showed that besides GTE and OLE, all plant products tested had limited effects on the *in vitro* viability of *N. perurans*

trophozoites. In a previous study, garlic *A. sativum* demonstrated *in vitro* activity against gill isolated amoebae, however, the *in vivo* efficacy was not investigated (Peyghan et al., 2008). Despite chili and black pepper performing significantly better ($P < 0.05$) against trophozoites, no further testing is warranted as efficacy was less than 50%.

In conclusion, CellTiter-Glo® presented the most advantages for high throughput screening of anti-*N. perurans* compounds. CellTiter-Glo® is least likely to interfere with investigations as the reagent is added at the end of the contact time. Due to the efficacy observed in this study, GTE and OLE were identified for further investigation.

Chapter Four

Effects of green tea extract (GTE) and olive leaf extract (OLE) on *Neoparamoeba perurans in vitro* and safety of GTE and OLE to Atlantic salmon *Salmo salar* (L)

Chapter 4 Effects of green tea extract (GTE) and olive leaf extract (OLE) on *Neoparamoeba perurans in vitro* and safety of GTE and OLE to Atlantic salmon *Salmo salar* (L)

4.1 Introduction

In vitro experiments in AGD research use either cultured amoebae or amoebae isolated from gills of diseased fish. This allows the study of the pathogen under standardised conditions to minimise any interference that may be present, simplifying the interaction between amoebae and anti-amoebic compound. Therefore, the experiment can focus on the identification of anti-amoebic compounds and quantification of its efficacy. Additionally, *in vitro* experiments reduce the need for laboratory personnel experienced in animal husbandry. Most importantly, it is a highly cost-effective method for screening of anti-amoebic compounds (Niles et al., 2009).

Green tea extract (GTE) contains polyphenols, vitamins, nitrogenous compounds, caffeine, inorganic compounds, lipids and carbohydrates (Chu and Juneja, 1997). Studies have shown that catechins, naturally occurring polyphenols, are the active components of GTE (Fraga et al., 1987; Vijaya et al., 1995; Jankun et al., 1997; Bohm, 1998; Huang et al., 1998; Jówko, 2015). The principal catechins present in GTE are epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate; with epigallocatechin gallate (EGCg) having the most bioactivity (Ma et al., 2000; Murakami et al., 2002). Catechins cause leakage of internal cellular contents by damaging cell membrane resulting in eventual cell lysis (Wesolowska et al., 2009). It was hypothesised that a critical concentration of EGCg bound to cell surface was required to cause cell lysis (Tamba et al., 2007).

Olive leaf extract (OLE), produced from the olive tree *Olea europaea* is rich in polyphenols such as oleuropein, rutin and verbacoside (Savournin et al., 2001). Similar to GTE, the main antioxidative and antimicrobial activities were associated with its polyphenols (Lee et al., 2009). The polyphenols of OLE that have physiological effects are hydroxytyrosol, tyrosol, caffeic acid, p-coumaric acid, vanillic acid, vanillin, oleuropein, luteolin, diosmetin, rutin, verbascoside, luteolin-7-glucoside, apigenin-7-glucoside and diosmetin-7-glucoside (Bianco and Uccella, 2000; Tasioula-Margari and Okogeri, 2001). *In vitro* studies have demonstrated that OLE has antimicrobial activity against pathogens such as *Helicobacter pylori*, *Campylobacter jejuni* and *Staphylococcus aureus* (Sudjana et al., 2009b), *Escherichia coli*, *Salmonella enteritidis*, *Enterobacter aerogenes*, *Bacillus cereus* and *Listeria monocytogenes* (Techathuvanan et al., 2014). The antimicrobial mode of action of OLE polyphenols is similar to other polyphenols (Devi et al., 2010) which is related to cell membrane damage (Carraro et al., 2014). When OLE was tested against *Acanthamoeba castellanii*, growth inhibition and amoebicidal activity were observed and activity appeared to be dose dependent (Sifaoui et al., 2013).

Despite GTE and OLE demonstrating activity against bacteria, viruses, ectoparasites including amoebae, they have not been tested against *Neoparamoeba perurans*. Furthermore, *in vivo* safety of GTE and OLE for Atlantic salmon, *Salmo salar*, is unknown. To investigate the effects of GTE and OLE, the toxicity of the extracts to gill isolated amoebae was tested *in vitro*. Toxicity was assessed using both manual counting and CellTiter-Blue® viability assay to determine if efficacy was affected by exposure time. A pilot study assessed *in vivo* safety of GTE and OLE.

4.2 Materials and methods

4.2.1 In vitro efficacy of GTE and OLE

Amoebae were isolated using a previously established method (Morrison et al., 2004). Briefly, gills were excised and dissected into individual gill arches, which were placed into 50 mL tubes and filled with 0.2 µm-filtered seawater. The tube was agitated to dislodge amoebae from the gills. The solution containing the suspended amoebae was poured into petri dishes and the amoebae allowed to attach at room temperature for 1 h. After the amoebae had attached, the plates were rinsed with 0.2 µm-filtered seawater and amoebae were dislodged using 0.05 % trypsin-EDTA (Sigma®). Cells were washed in 0.2 µm-filtered seawater and concentrated by centrifugation (450 x *g* for 5 min, Eppendorf Centrifuge 5810 R) and counted. Guidelines for OLE and GTE exposure for salmonids in seawater were not available, therefore this experiment used GTE exposure time and concentration based on what Suzuki et. al., 2006 used for GTE bath treatment of other salmonids in freshwater. OLE has not been tested as a bath treatment in fish, therefore concentrations tested in this experiment were based on an arbitrary concentration of 1.25 g L^{-1}. Exposure time was based on the longest exposure time used by Suzuki et. al., 2006.

4.2.2 Viability using manual counting

Amoebae were re-suspended in 0.2 µm-filtered seawater and transferred into individual wells of a 96-well plate (100 cells per well). The plate was incubated at 18°C for 1 h to allow amoebae to adhere to the wells. Seawater was removed and replaced with 200 µL of selected concentrations of plant extracts (prepared using 0.2 µm-filtered seawater, 35ppt)

for the prescribed exposure time. 0.2 µm-filtered seawater was used as negative control and 1% formalin was used as positive control. Selected concentrations of GTE (Bulk Nutrients, EGCg 40%) were 300, 150, 75, 38, 19 and 9 mg L^{-1}, had an exposure time of 30 min. Selected concentrations of OLE (Nature's Care Manufacture Pty. Ltd., 1.7 % oleuropein) were 12,500, 6,250, 1,562, 780 and 390 mg L^{-1}, had an exposure time of 60 min. Immediately following incubation, all seawater containing the plant extract and detached amoebae were removed from individual wells and transferred into 1 mL microcentrifuge tubes containing 500 µL of 0.2 µm-filtered seawater. Any amoebae that remained attached were detached using 100 µL of 0.05 % trypsin and added into corresponding microcentrifuge tubes. The microcentrifuge tubes were vortexed and amoebae concentrated by centrifugation (13,000 x *g*, 15 s, Eppendorf 5415D), which removed most of the supernatant, leaving behind approximately 20–30 µL of concentrated amoebae.

Viability was assessed in triplicates by the inclusion of a vital dye (Neutral Red, Sigma). 20 µL of Neutral Red solution was added to 20 µL of amoebae suspension and incubated at 18 °C for 20 min. Excess dye was removed by the addition of 800 µL of 0.2 µm-filtered seawater and centrifuged at 13,000 x *g* for 15 s (Eppendorf 5415D). 800 µL of supernatant containing the excess dye was removed. Viability was determined by microscopic examination using an inverted microscope at 40 x magnification, cells that had internalized the dye were considered viable. Duration of counting of amoebae in each well was not standardised between each count.

4.2.3 CellTiter-Blue® cell viability assay

For this study, CellTiter-Blue® Cell Viability Assay (Promega©) was modified (Crosbie, pers. comms.) from the manufacturer's protocol as follows. Total individual well volume was made up of 10 µL of live amoebae suspension (n = 100), 20 µL of formalin-inactivated amoebae suspension (n = 300), 20 µL of CellTiter-Blue® Cell Viability Assay reagent and 50 µL of plant extract at selected concentrations. Viability estimation was performed in triplicates. 0.2 µm-filtered seawater was used as negative control and 1% formalin was used as positive control. The addition of formalin-inactivated amoebae was essential for the stimulation of metabolic activity in viable cells in order to generate a signal. Selected concentrations of GTE and OLE were 300, 150, 75, 37, 19 and 9 mg L^{-1}, and 12,500, 6,250, 1,562, 780 and 390 mg L^{-1} respectively. Formalin-inactivated amoebae were prepared as follows: 10 µL of 10 % formalin was added to 990 µL of amoebae suspension and incubated at 18 °C for 30 min and washed twice by centrifuging at 13,000 x *g* for 15 s and removing supernatant after adding 900 µL of 0.2 µm-filtered seawater. The plate was read after incubation for 16 h at 18 °C using Tecan© Infinite 200 Pro plate reader at 560 nm (excitation) and 590 nm (emission) wavelengths.

4.2.4 Statistical Analysis

One-way ANOVA (SPSS® Version 20; IBM®) was used to determine the effect of GTE and OLE on the viability of *N. perurans* trophozoites *in vitro*. Data were tested for normality and homogeneity using Shapiro–Wilk test and Levene's test respectively. Data were subjected to transformation if normality was not met and the Welch test was used when homogeneity was not met. Tukey's HSD was used for comparisons of means where assumptions of

normality and homogeneity were met. $P < 0.05$ was adopted for the rejection of the null hypothesis.

4.2.5 Pilot safety assessment of GTE and OLE

Only 2 fish were used per concentration to minimise the number of fish that might be subjected to any adverse effects. All fish in this trial were approved for experimentation by the University of Tasmania, Australia (Animal Ethics Committee Permit No. A13840). Atlantic salmon (n = 8, mean weight ± SE, 150 g ± 1.49) were obtained from the Aquaculture centre at the University of Tasmania. Fish were held at 14 °C and under 14L:10D photoperiod at 30 ppt salinity in a 4000 L recirculating system. Fish had been acclimated to seawater over two weeks with salinity being raised by 5 ppt every two days.

OLE was tested at higher than effective *in vitro* concentration to assess the safety of OLE *in vivo*. A bath containing OLE was prepared by dissolving 60 g OLE (Nature's Care Manufacture Pty. Ltd.) in 40 L seawater (35 ppt, 13 °C, >90 % O_2) to give a concentration of 1500 mg L^{-1}; the bath was filtered (dacron filter mat) before addition of fish. Two fish were added to the OLE bath for 1 h. OLE bath was constantly aerated and dissolved oxygen monitored using a dissolved oxygen probe. Immediately following bath treatments in OLE, fish were dip-netted from the treatment tank and quickly transferred into an aerated circular 100 L tank filled with fresh seawater for observations after the bath. Gill baskets of all fish were excised 24 h after the bath, briefly rinsed in SW, fixed in SW Davidson's fixative and transferred to 70 % ethanol 24 h after fixation for histology (2nd left anterior hemibranch). 2 fish were used in the safety assessment of OLE.

A bath containing GTE (Bulk Nutrients, 40 % Epigallocatechin-3-gallate) and seawater (35 ppt, 13 °C, >9 0% O_2) was prepared by dissolving 12 g GTE in 40 L seawater to give a concentration of 300 mg L^{-1}. This concentration was selected as it had demonstrated *in vitro* effects against *N. perurans* trophozoites. Two fish were added to the GTE bath for 30 min. Immediately following bath treatment fish were dip-netted from the treatment tank and quickly transferred into an aerated circular 100 L tank filled with fresh seawater for observations after the bath. Dead and moribund fish (euthanized using 40 mg L^{-1} clove oil in seawater) had the entire gill basket excised, briefly rinsed in SW, fixed in SW Davidson's fixative and transferred to 70 % ethanol 24 h after fixation for histology (2nd left anterior hemibranch).

The following concentrations of GTE, 150 and 75 mg L^{-1}, were tested to determine safe *in vivo* concentration. The GTE bath was diluted 50 % by decanting 20 L of the original bath and 20 L of seawater added to give 150 mg L^{-1} concentration. Two fish were added to the GTE bath for 30 min. After bath treatment, fish were treated as described in previous concentrations. The GTE bath was diluted 50% by decanting 20 L of the current bath and 20 L of seawater added to give 75 mg L^{-1} concentration. Two fish were added to the GTE bath for 30 min. After bath treatment, fish were treated as described in previous concentrations. During the bath, fish were monitored visually for signs of distress such as rapid gill movements, unable to maintain equilibrium and erratic swimming. GTE bath was constantly aerated and dissolved oxygen monitored using a dissolved oxygen probe. 6 fish were used in the safety assessment of GTE.

4.3. Results

4.3.1 In vitro efficacy of GTE and OLE

Both OLE and GTE were observed to have anti-*N. perurans* effects *in vitro*. When amoebae were exposed to either GTE or OLE, reduction in viability was observed using both manual counting and CellTiter-Blue®. However, the results obtained using manual counting and viability assay were different. Viability assay indicated lower viability as compared to manual counting when amoebae were tested with similar concentrations of extracts. When amoebae were exposed to OLE, CellTiter-Blue® indicated 8 % ± 0.23 viability after exposure at 390 mg L^{-1}, whereas manual counting indicated viability of 38 % ± 0.31 (Figure 4.1). Similarly, when amoebae were exposed to GTE, CellTiter-Blue® indicated the loss of viability at all concentrations tested, whereas manual counting indicated viability of 33 % ± 0.34 and higher when amoebae were exposed to GTE concentrations of 75 mg L^{-1} and lower (Figure 4.2).

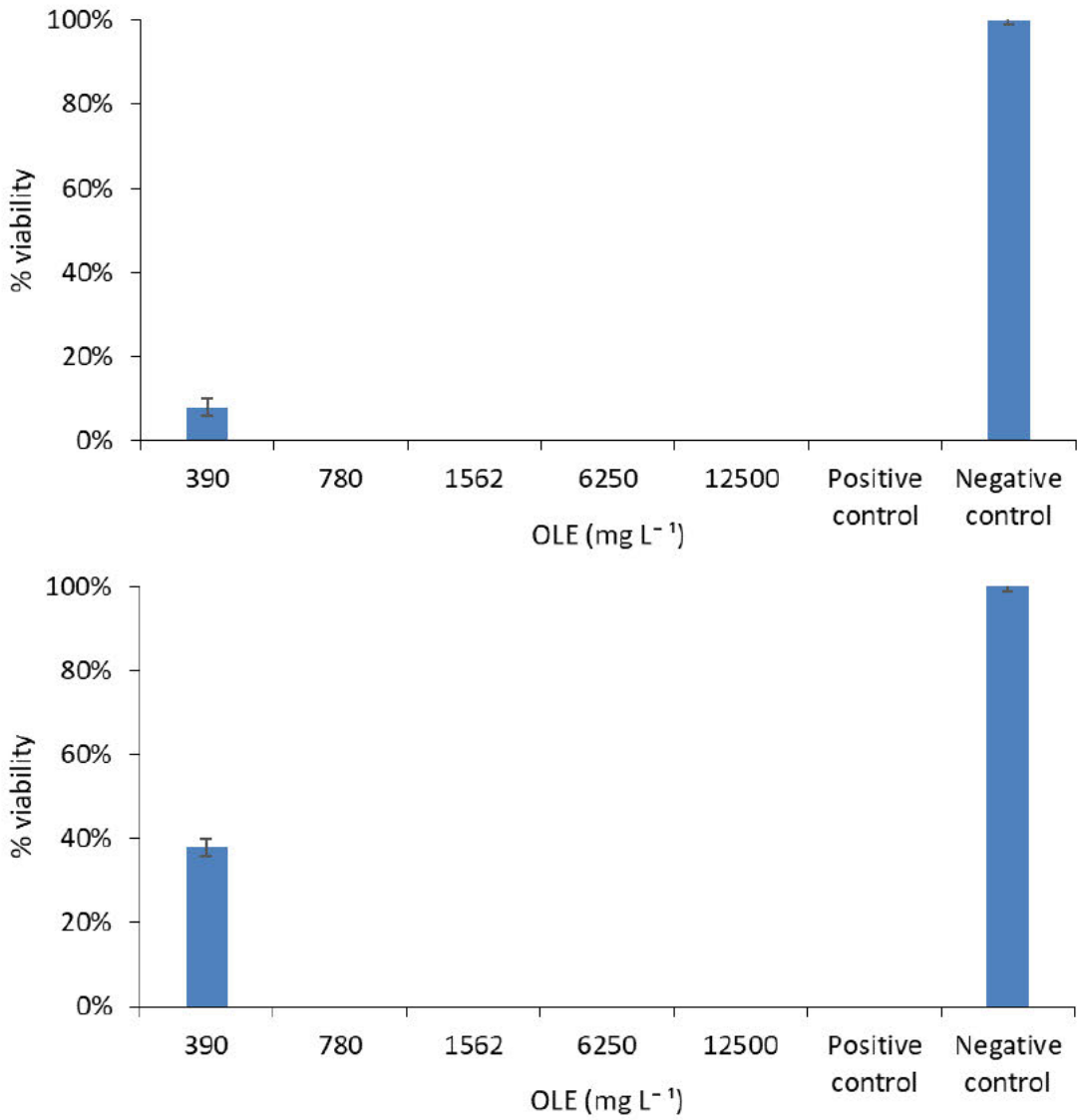

Figure 4.1. Viability of amoebae after exposure to OLE. Top graph illustrates viability of amoebae as determined by CellTiter-Blue® viability assay after 16 h incubation with OLE. Bottom graph illustrates viability of amoebae as determined by neutral red manual counting method after 1 h incubation with OLE. Results are presented as means ± standard error.

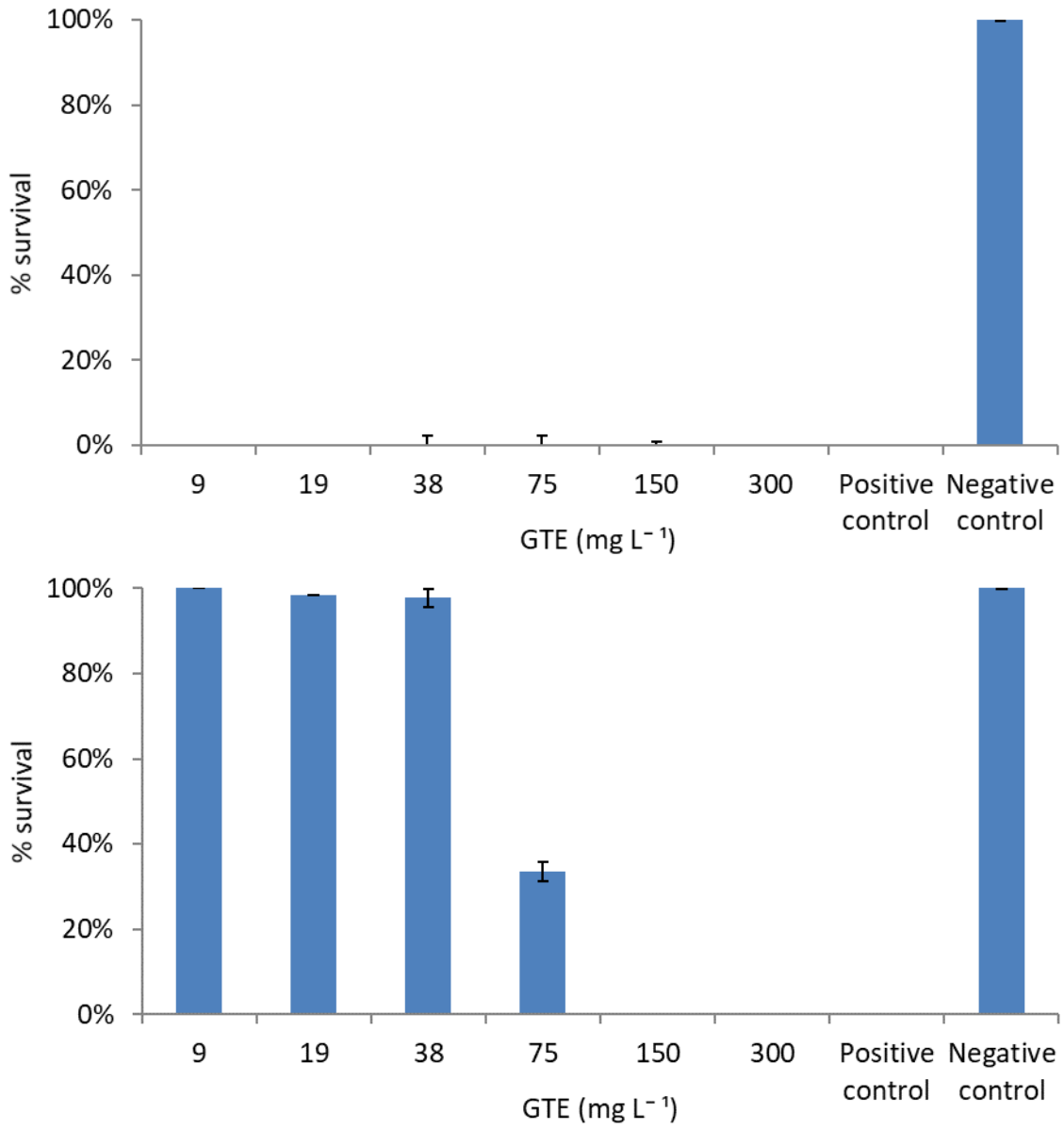

Figure 4.2. Viability of amoebae after exposure to GTE. Top graph illustrates viability of amoebae as determined by CellTiter-Blue® viability assay after 16 h incubation with GTE. Bottom graph illustrates viability of amoebae as determined by neutral red manual counting method after 30 min incubation with GTE. Results are presented as means ± standard error.

When viability was assessed using manual counting, exposure of amoebae to GTE at 150 mg L^{-1} and 75 mg L^{-1} for 30 min resulted in 100 % and 67 % ± 0.34 loss of viability respectively. This was significantly lower than all lower concentrations tested (F = 65.485, df 4, 10, P < 0.05). Exposure of amoebae to OLE at 780 mg L^{-1} for 1 h resulted in 100 % loss of viability. When amoebae were exposed to OLE at 390 mg L^{-1} for 1 h, 38 % remained viable; this was significantly lower compared with the control (F = 902.455, df 1, 4, P < 0.05).

4.3.2 Pilot study: Olive leaf extract

During OLE bath, no signs of distress were observed in fish and no mortality was recorded 24 h after treatment. This was the end point of the experiment and fish were euthanised for histology. Histological examination of gills 24 hours after treatment indicated no adverse reactions and the gills appeared healthy and normal (Figure 4.3).

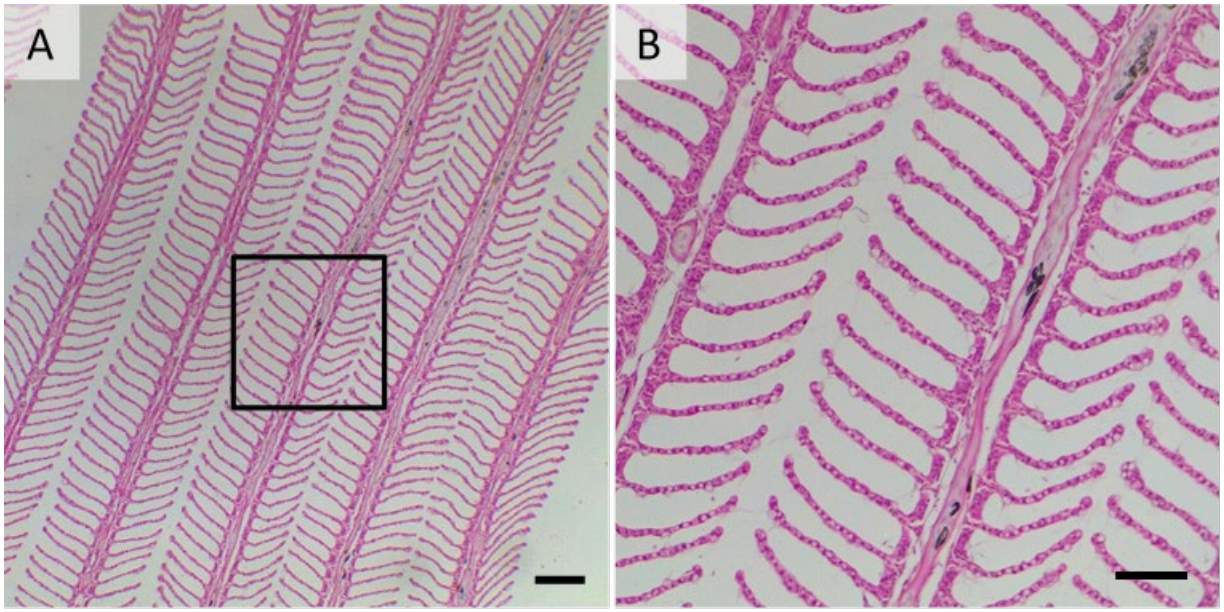

Figure 4.3 Histological images of salmon gill after treatment with OLE. (A) Healthy gill 24 h after OLE treatment. Bar = 200 µm. (B) Enlarged boxed section of healthy gill. Bar = 50 µm.

4.3.3 Pilot study: Green tea extract

When fish were introduced to 300 and 150 mg L^{-1} GTE baths, they were swimming erratically with rapid opercular movements. After 5 min, erratic swimming movements ceased and fish were able to maintain equilibrium and position in the water column. After transfer to the observation tank, fish from both treatments were not swimming but were able to maintain equilibrium and position in the water column. After 5 min in the observation tank, fish ceased opercular movements and were unresponsive to external stimuli. Fish were determined to have died upon visual observation. Fish subjected to 75 mg L^{-1} GTE bath exhibited behaviour similar to previously observed during treatments at higher concentrations. After transfer to the observation tank, fish had normal swimming rates and opercular movements, and were able to maintain equilibrium and position in the water column. Observations 20 min after treatment indicated no change and fish were responsive to external stimulus. However, fish exposed to 75 mg L^{-1} GTE did not survive overnight and mortality was recorded 16 h after treatment. Histological examination (post mortem) of gill sections from fish treated with all concentrations of GTE presented with epithelial lifting and necrosis (Figure 4.4). Necrosis and epithelial lifting observed in the gill section of 75 mg L^{-1} GTE treated fish were most likely due to delayed sampling post mortem as fish might have died during the overnight recovery period.

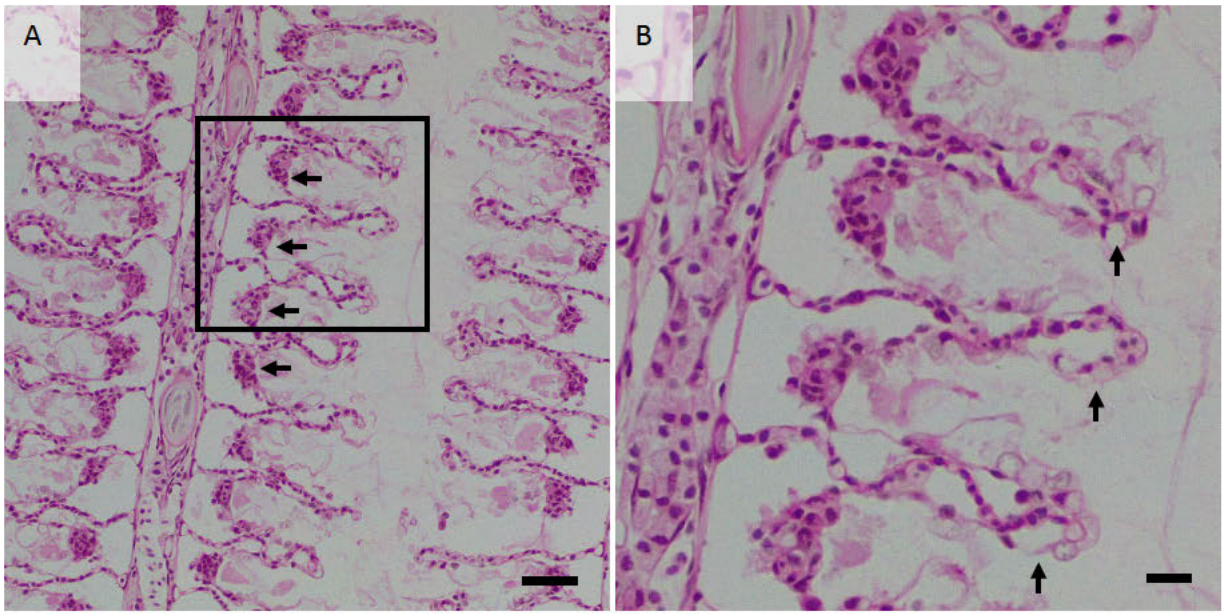

Figure 4.4. Histological image of salmon gill after treatment with 75 mg L^{-1} GTE. (A) Gill of salmon after treatment; notice lifting of filamental epithelium indicated by arrows. Bar = 50 µm. (B) Enlarged boxed section, notice lifting and necrosis of lamellar epithelium indicated by arrows. Bar = 15 µm.

4.4 Discussion

4.4.1 In vitro efficacy of GTE and OLE

The study demonstrated that GTE and OLE were successful *in vitro* at reducing the number of viable amoebae relative to controls, regardless of methods used to assess viability and incubation time. When viability was assessed by manual counting, results were similar to results reported in Chapter 3 using CellTiter-Glo®. According to manual counting, the lowest concentration of GTE capable of killing > 50% of amoebae was 75 mg L^{-1} and the lowest concentration capable of killing all amoebae was 150 mg L^{-1}. GTE has been used successfully *in vivo* as a bath treatment on chum salmon, *Oncorhynchus keta*, and masu salmon, *O. masou*, against *Ichthyobodo necator* and toxicity against the host was observed at twice the

concentrations tested in this study (Suzuki et al., 2006). The active constituent of GTE, EGCg, also exhibited activity against the protozoan parasite *Trypanosoma brucei* during *in vitro* testing at half the concentration tested in this study (Vigueira et al., 2012).

Results from this study indicated that cell viability estimation during GTE exposure was different between manual counting and CellTiter-Blue® viability assay. This might be due to incompatibility between GTE and CellTiter-Blue® reagent, thus making this viability assay unsuitable for use with GTE. Based on a model built to study the mode of action of EGCg, the observed antiprotozoal effect results from its ability to cause cell lysis when bound to the cell surface (Tamba et al., 2007; Wesolowska et al., 2009). Therefore, a more appropriate cell viability assay would be an assay that measures cell membrane damage e.g. CytoTox-ONE™. Similarly, a more suitable method of manual counting would be a dye exclusion method, such as Trypan Blue vital stain, where viability is determined by cell membrane integrity.

Similar to GTE, when the viability results of OLE determined by manual counting were compared with the results from CellTiter-Blue® assay, lower viability was observed using CellTiter-Blue® as compared to manual counting. This suggests a relationship between time and efficacy of OLE. Manual counting had an exposure time of 60 min compared with 16 h during CellTiter-Blue® assay. This relationship between time and efficacy was also seen in Chapter 3. When OLE *in vitro* efficacy was determined using CellTiter-Glo®, viability after 2 h exposure at 390 mg L^{-1} was 21 % (Chapter 3) as compared to 8 % after 16 h exposure (this Chapter).

Based on the results of this study, GTE and OLE are possible candidates for further investigation as novel bath treatments against AGD. The use of *in vitro* testing has facilitated the screening of plant extracts for activity against *N. perurans*. However, it is important to understand the mode of action of the substance being tested. As both GTE and OLE are crude extracts containing a mixture of compounds, it is unknown if the compounds have any synergistic effects between them. Future experiments should be conducted to understand the mode of action of the extracts.

4.4.2 Pilot study

Results of the pilot study indicated that GTE is not suitable as bath treatment for Atlantic salmon at concentrations that were effective against *N. perurans* trophozoites *in vitro*. Atlantic salmon smolts were unable to survive GTE bath treatment at concentrations of 300, 150 and 75 mg L^{-1} for 30 min. In contrast, chum salmon *Oncorhynchus keta* and masu salmon *Oncorhynchus masou* fry and alevin were able to tolerate GTE bath treatment of 300 mg L^{-1} for 30 min and 3000 mg L^{-1} for 15 min (Suzuki et al., 2006).

While GTE concentrations used were similar to those reported by Suzuki et al. (2006), experimental conditions were different. Firstly, when GTE was tested on *O. keta* and *O. masou*, the experiment was conducted in freshwater (Suzuki et al., 2006). Secondly, the GTE used in both experiments were different, Suzuki et al. (2006) used GTE with 37 % catechin and this study used GTE with 90 % catechin. GTE is a mixture of compounds and it is unknown if the compounds within GTE act individually or in synergy, therefore it is difficult to draw a comparison against both studies. It has been reported that the composition of GTE varies between batches (Lee et al., 2014). This meant that the GTE used in both studies

were likely to be compositionally different and therefore could not be compared. Furthermore, GTE was not safe to use on Atlantic salmon even at the lowest concentration tested which is lower than what was established as safe by Suzuki et al. (2006). Finally, this experiment was conducted in seawater on seawater acclimated fish whereby (Suzuki et al. (2006)) conducted their experiment on fish held in freshwater water. Salinity would have had an effect on GTE tolerance as it was demonstrated that organisms in saltwater were more sensitive to phenols as compared to organisms in freshwater (Wheeler et al., 2002). Most likely it was the difference in catechin concentrations that affected the safety of GTE at concentrations tested.

This pilot study established that OLE was safe for use on Atlantic salmon at the concentrations tested. During the pilot study, fish did not exhibit signs of distress both during and after bath exposure. When fish were left for 24 h to observe recovery after the bath, no signs of distress were noted. This pilot study could be further strengthened if blood cortisol levels were evaluated before and after OLE bath. Cortisol is an easily measured component of the primary stress response and it is used as an indicator of stress when evaluating fish welfare (Ellis et al., 2012). This stress response could then be compared against freshwater bathing as stress is detrimental to production issues such as growth and disease susceptibility (Pickering, 1992). Cortisol levels were not measured as this experiment was designed primarily to evaluate the safety of the OLE bath. Histological examinations of gills 24 h after bath indicated no adverse reactions or indications of any safety concerns. It was determined that the concentrations tested were safe and OLE was suitable for use in the following investigation (Chapter 5).

This study had shown that GTE and OLE were efficacious against *N. perurans in vitro* and only OLE was safe to use on fish at effective *in vitro* concentrations. Further investigation of OLE treatment administrated as a therapeutic bath against AGD should be explored to elucidate efficacy against AGD *in vivo*. Additionally, two aspects of investigating any new treatments were highlighted in this study. Firstly, the selection of a suitable viability assay is dependent on the mode of action of the compound tested. Secondly, a pilot study to assess *in vivo* safety should be conducted before proceeding to a complete *in vivo* experiment.

Chapter Five

Efficacy of olive leaf extract as a bath treatment for amoebic gill disease in Atlantic salmon *Salmo salar* (L.)

Chapter 5 Efficacy of olive leaf extract as a bath treatment for amoebic gill disease in Atlantic salmon *Salmo salar* (L.)

5.1. Introduction

Aquaculture entails intensive animal husbandry, keeping large numbers of fish in a comparatively small space can increase the risk of disease occurrence (Stoffregen et al., 1996). Under such circumstances, parasites are provided with ideal conditions for disease to spread and progress rapidly, this combined with reduced water quality results in substantial mortalities (Schmahl et al., 1989). Hence, the need for control of AGD is of high importance as mortality due to disease can be as high as 82% if left untreated (Steinum et al., 2008; Rodger, 2014; Powell et al., 2015). Investigations on AGD mitigation had mainly been focused on chemical based anti-protozoal compounds and freshwater (Howard and Carson, 1994; Howard, 2001; Munday and Zilberg, 2003; Florent et al., 2007c; b). Chemotherapeutants had been successfully administered orally in-feed to treat parasitic disease caused by endoparasites such as nematodes and cestodes, whereas treatment of ectoparasites such as trichodinads and monogeneans requires bath administration (Noga, 2011). In addition to successful applications of chemotherapeutants, the use of plant extracts could potentially reduce the cost of treatment and be more environmentally friendly as they are likely to be more biodegradable (Blumenthal et al., 2000; Logambal et al., 2000; Olusola et al., 2013).

Terrestrial plants are known to produce phenolic compounds with oxidative, antibacterial and antiviral activities, beneficial or otherwise, for human or animal health (Meot-Duros and Magne, 2009). Phenolic compounds are secondary metabolites that are formed by three

biosynthetic pathways: polyketide pathway, succinylbenzoate pathway and mevalonate pathway (Knaggs, 2001; Bhattacharya et al., 2010). Phenols are a class of chemical compounds consisting of a hydroxyl group bonded directly to an aromatic hydrocarbon group (Bravo, 1998). Besides contributing to the colour and sensory characteristics of plants (Alasalvar et al., 2001), phenolic compound such as oleuropein are known to have antiparasitic activity (Jiang et al., 2008; Elamin and Al-Maliki, 2014; Kyriazis et al., 2016; Kyriazis et al., 2017).

Despite salmonids being higher tropic level carnivorous species (Marandel et al., 2018), incorporation of plant extracts into fish diets have been shown to enhance disease resistance (Abdel-Tawwab et al., 2010; Harikrishnan et al., 2011a; Sheikhzadeh et al., 2011). When administered as a topical bath treatment, plant products have also demonstrated therapeutic effects against external parasites (Suzuki et al., 2006; Kumar et al., 2012b). Furthermore, the use of plant extracts is less likely to cause drug resistance in parasites due to the high diversity of plant extract constituents (Blumenthal et al., 2000; Logambal et al., 2000; Olusola et al., 2013).

The most significant disease affecting the Tasmanian Atlantic salmon industry is a parasitic disease known as amoebic gill disease (AGD) caused by *Neoparamoebae perurans* (Young et al., 2008b). *N. perurans* is a free-living marine protozoa that attaches itself to the gills of Atlantic salmon (Adams and Nowak, 2003). Macroscopically, AGD presents as raised, white mucoid patches (Alexander, 1991). In histology sections, AGD presents as focal and multi-focal hyperplasia of the primary and secondary lamella (Adams et al., 2004). Lamellar fusion is also a prominent observation often resulting in the formation of cystic spaces (Munday et

al., 2001). The parasitic amoebae, *Neoparamoeba*, could be seen on the gills of previously naïve fish 12 hours after exposure to fish affected by AGD (Adams and Nowak, 2004). Seven days after exposure, multifocal hyperplasia and lamellar fusion involving up to 15 gill lamellae were observed (Zilberg and Munday, 2000). Amoebae are often observed adhered to or in close proximity to lesions and sometimes encased with interlamellar vesicles or cysts (Adams and Nowak, 2001; Parsons et al., 2001b). Immediately after freshwater bathing of AGD affected fish, lesions were cleared of attached trophozoites and cellular debris (Adams and Nowak, 2004). These histological features are routinely used to gauge AGD severity and treatment effectiveness during experiments (Parsons et al., 2001b; Adams et al., 2012).

Olive leaf extract (OLE) is the extract of leaves of the olive tree *Olea europaea* which is known to have antiparasitic properties (Zunin et al., 1995; Guarrera, 1999). The beneficial properties associated with OLE are mainly derived from its polyphenol constituents such as oleuropein, rutin ,verbacoside and triterpenic acid (Benavente-Garcıa et al., 2000). OLE polyphenols act directly upon enzymes, proteins, receptors and signalling pathways in human brain (Halliwell, 2001; Williams et al., 2004) as well as interference in gene expression of chromatin (Ayissi et al., 2014). This results in reduced cell viability and induction of thiol group modifications, reactive oxygen species, expression of y-glutamylcysteine synthetase, pAkt and heme oxygenase-1 (Acquaviva et al., 2012). Antimicrobial activity of OLE was demonstrated in pathogenic bacteria and virus such as *Listeria monocytogenes, Staphylococcus aureus* (Techathuvanan et al., 2014) and viral haemorrhagic septicaemia rhabdovirus (Micol et al., 2005). Furthermore, Sifaoui et al. (2013) reported antiparasitic effects of OLE against *Acanthamoeba castellanii* Neff trophozoites.

Olive oil wastewater, a by-product of olive oil production with similar chemical composition as OLE, was studied for their antioxidant properties (De Lucia et al., 2006; Soni et al., 2006b; Di Benedetto et al., 2007), antiparasitic properties (Zunin et al., 1995; Guarrera, 1999) and their effects on human health (D'Angelo et al., 2005a; Singh et al., 2008). Studies of effects of olive leaf and similar by-products of olives on fish have been mainly done to improve sensory perception. When fish were fed diets formulated with the addition of olive oil wastewater, fish fillets had higher antioxidant levels and better sensory qualities (Sicuro et al., 2010b) which resulted in delayed development of oxidation leading to better conservation of fillets (Sicuro et al., 2010a). Similarly, when fish were fed diet incorporated with olive leaf powder, microstructural observations of fish muscle showed higher endomysium structure rigidity resulting in an improved final product (Arsyad et al., 2018) which had lower dark muscle discoloration resulting in better flesh quality (Oyama et al., 2010).

Given the antimicrobial (Micol et al., 2005; Sifaoui et al., 2013; Techathuvanan et al., 2014), antiparasitic properties (Zunin et al., 1995; Guarrera, 1999) and *in vitro* amoebicidal activity of OLE demonstrated in the previous chapters (Chapter 3 & 4) it is hypothesised that topically treating fish with OLE may ameliorate clinical and histopathological signs of branchialitis caused by infection with *N. perurans*. Therefore, the aim of this study was to histologically assess the efficacy of bathing Atlantic salmon affected by experimentally induced AGD with OLE in seawater.

5.2. Material and methods

Experimentation in this chapter was approved by the University of Tasmania's animal ethics committee (Approval # A0013840). Atlantic salmon smolts (n=180, mean weight ± SE 147 g ± 1.51) were obtained from the University of Tasmania's Aquaculture Centre. Fish were held under 14L:10D photoperiod in a 4000L recirculating freshwater system maintained at 14 °C. The fish were allocated into nine tanks (n=20 per tank) of a modular recirculating freshwater system (consisting of 12 x 350 L Reln style tanks) equipped with mechanical filtration, bio-filtration, foam fractionation and UV disinfection. Water flow to the other 3 tanks was switched off. This modular system was successfully used by Adams et al. (2012). Water was batch exchange every 3 days to maintain water quality, the exchange rate was equivalent to 10 % per day and fish were fed a commercial ration (Skretting) at a rate of 0.75 % bodyweight daily. Water quality during the experiment was maintained at pH 7.8 - 8, NH_3 <0.25 mg L^{-1}, NO_2 <2 mg L^{-1} and NO_3 < 80 mg L^{-1}. Fish were gradually acclimated to seawater (35 ppt) over a period of 3 weeks and the temperature was kept at 15 °C (± 0.5 °C). Prior to exposing fish to *N. perurans* trophozoites, it was noted that a mild ulcerative dermatitis was beginning to establish within the population (10.7% of fish affected). Affected fish were observed to have focal areas of <1cm diameter of raised and/or missing scales on the flanks. Consequently, fish were treated in a freshwater bath for two hours and held at 25 ppt salinity for 25 days at which point no further signs of dermatitis were observed. At this point, salinity was raised to 35 ppt over a period of 3 days.

5.2.1 Exposure of fish to N. perurans trophozoites

Following acclimation, fish were exposed to *N. perurans* trophozoites (250 trophozoites L^{-1}). The amoebae were isolated from donor fish using the method described by Morrison et al. (2004). Fish were then exposed to trophozoites (250 trophozoites L^{-1}) twice more at days 10 and 18 after initial exposure. No further health issues aside from AGD were observed during the remaining duration of the experiment. Fish were exposed to trophozoites for 24 days prior to treatment.

5.2.2 Determination of OLE treatment efficacy in vivo

During treatment, fish were divided into 3 groups (20 fish per group, 3 replicate groups) and randomly allocated a bath treatment of either OLE (Nature's Care Manufacture Pty Ltd) in SW, freshwater (de-chlorinated mains water, 15 °C, pH 8, hardness 7 mg L^{-1}) or seawater (0.2 µm-filtered, ambient temperature, 15 °C) only. Fish were randomly allocated a bath treatment by assigning each tank a number, writing the numbers onto individual pieces of paper, placing all the pieces of paper into a cup and randomly picking out a number from it. The first 3 tanks picked were given OLE treatment, the next 3 FW treatment and the remaining 3 SW treatment. After treatment, fish were placed into a system similar to the one they were in previously (Figure 5.1). How the fish were allocated after treatment was unlikely to affect the experiment as the tanks are all connected to a central sump where the overflow from all the tanks is mixed and recirculated back into the tanks. This tank system has been previously used by Adams et al. (2012) to elucidate the effects of hydrogen peroxide against fish with AGD. Olive leaf extract bath was prepared by dissolving 78 g of OLE in 100 L of seawater (35 ppt, 15 °C, >90 % O_2 saturation) to give a concentration of 780

mg L^{-1}. Fish were first crowded by dropping the water level of the holding tank, quickly transferred using dip net to the OLE bath for 1 h. Likewise, fish were treated with freshwater bath for 2 h (as a positive control) and negative control (SW bath) fish were treated with seawater bath for 1 h. Five fish from each tank were removed before the bath. These fish were euthanised (40 mg L^{-1} clove oil in seawater), measured, visually inspected for gross abnormalities and the gills removed and fixed in SW Davidson's fixative for histology. Similarly, immediately after each bath, five fish were removed and the gills collected as described above. Following the bath, all fish were dip–netted from their respective treatment tanks and transferred to an identically configured modular recirculating system (as described above). A further group of sentinel fish (n=12, mean weight ± SE, 345 g ± 21) were introduced to one of the three remaining tanks to determine to what extent fish would be susceptible to horizontal transmission of AGD after bath treatment. This meant that in the 12-tank system, 10 tanks were running and 2 were left empty with the water switched off. Remaining fish from all tanks were then sampled as above seven days after the bath.

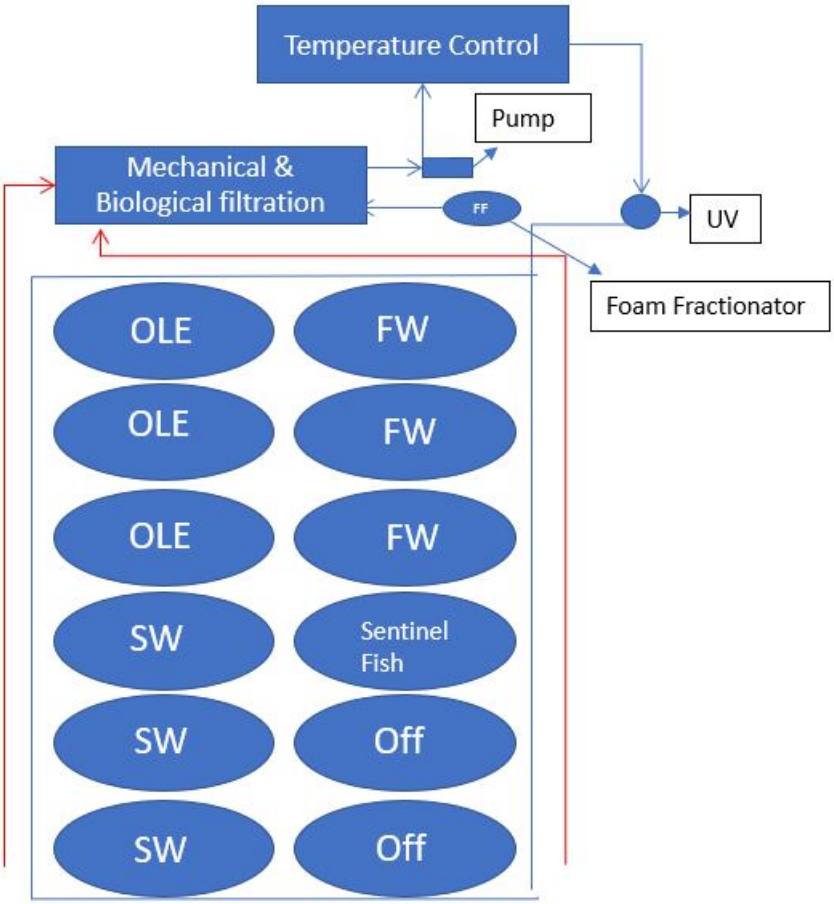

Figure 5.1 Drawing of experiment system. All tanks were connected to a central sump equipped with mechanical and biological filtration, temperature control, foam fractionator and UV disinfection.

5.2.3 Fish sampling

During sampling, the entire gill basket was excised, briefly rinsed in SW, fixed in SW Davidson's fixative and transferred to 70 % ethanol 24 h after fixation for histology. Histological analysis of the 2nd left anterior hemibranch was undertaken to determine the percentage of filaments with hyperplastic lesions. Each filament was inspected for the presence and size (interlamellar units, ILU) of hyperplastic lesions and the presence or

absence of *N*. *perurans* trophozoites upon each filament. Prevalence is the percentage of a population that have a specific characteristic in a given sampling point. For example, the prevalence of fish positive for *N. perurans* is the percentage of fish within each tank that had amoebae that were observed in close proximity of hyperplastic lesion. This is presented as mean prevalence ± SE of the 3 replicate tanks. Figure 5.1 lists all the prevalence of the AGD indices measured and their definitions.

Table 5.1 List of AGD indices measured and how each index is defined.

AGD index	Definition
Prevalence of fish positive for *N. perurans*	% of fish in each treatment group with amoebae that were observed in close proximity of hyperplastic lesion
Prevalence of fish with hyperplastic lesions	% of fish in each treatment group with hyperplastic lesions
Prevalence of filaments with hyperplastic lesions	% of filaments with hyperplastic lesions in each fish of each treatment group
Prevalence of lesions colonised with one or more amoebae	% of lesion of each treatment group that had one or more amoebae that were observed in close proximity

Prevalence of filaments with hyperplastic lesions colonised by one or more amoebae	% of filaments with hyperplastic lesions that had amoebae that were observed in close proximity
Lesion size	Number of interlemallar units

5.2.4 Statistics

Two-way ANOVA (SPSS® Version 20; IBM®) was used to determine the interaction between each measurement variables (Table 5.1) and two orthogonal factors (treatment and time). Percentage data were subjected to arcsine transformation before analysis. Tukey's HSD was used for comparisons of means where assumptions of normality (Shapiro–Wilk test) and homogeneity (Levene's test) were met. $P < 0.05$ was adopted for rejection of the null hypothesis. Sentinel fish were not included in these analyses.

5.3. Results

Fish sampled prior to treatment displayed gross signs indicative of AGD prior to treatment and were verified histologically (Figure 5.2). The severity of AGD of fish sampled was classified as light in a clinical sense (mean 7.88% ± 1.04 filaments with lesions, range 1 – 17 %, Figure 5.3). Hyperplasia and lamellar fusion indicative of lesions arising from amoebic infection were present in histological sections (Figure 5.2).

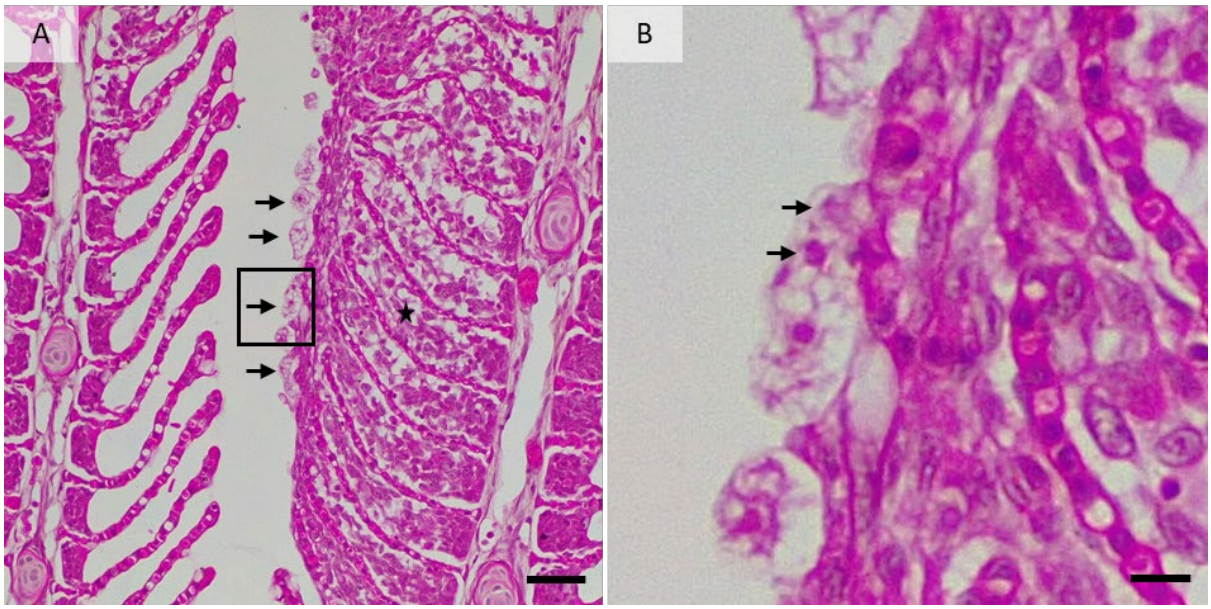

Figure 5.2. Histological images of salmon gills before bath treatment. (A) Gill associated Paramoebae present on lesions. Lamellar fusion (black star) and parameoboid trophozoites present upon surface of hyperplastic lesion indicate by arrows. Bar = 50 µm. (B) Enlarged section from (A) showing the nucleus and endosymbiont of a trophozoite (black arrows). Bar = 10 µm.

No amoebae were observed upon the gill lesions of fish sampled immediately after FW bathing. When fish were sampled immediately after treatment, FW and OLE treated fish had a significantly lower prevalence of gill filaments with lesions colonised by Paramoebae as compared to SW treated fish ($F = 38.671$, df 2,156, $P < 0.001$). When fish were sampled seven days after treatment, OLE treated fish had a significantly lower prevalence of gill filaments with lesions colonised by Paramoebae when compared against SW treated fish ($P < 0.001$). When fish were sampled seven days after treatment, there was no significant difference ($P > 0.05$) in the prevalence of gill filaments with lesions colonised by Paramoebae between fish that were treated with OLE and FW. When fish were sampled

after treatment, FW treated fish had the lowest prevalence of gill filaments with lesions colonised by Paramoebae both immediately after and seven days after treatment when compared against OLE and SW. When fish were treated with either OLE or FW, the prevalence of gill filaments with lesions colonised by Paramoebae was significantly lower ($P < 0.001$) both post treatment and 7 days post treatment. When fish were treated with SW, there was no significant difference ($P > 0.05$) in the prevalence of gill filaments with lesions colonised by Paramoebae post treatment and seven days post treatment (Figure 5.3).

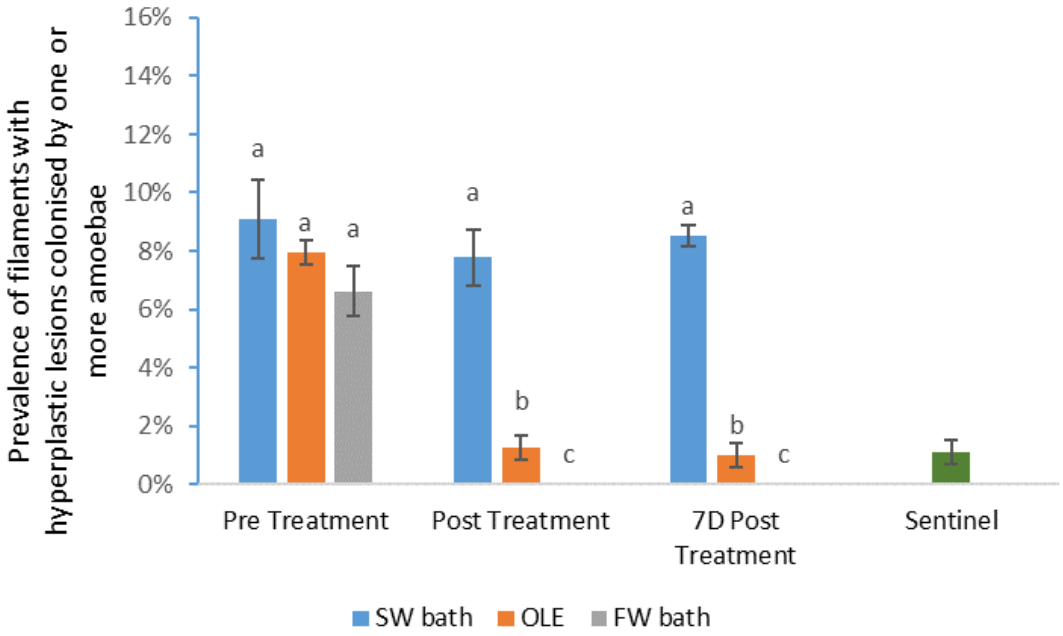

Figure 5.3. Prevalence of gill filaments with lesions colonised by Paramoebae. Results were presented as means ± SE, sentinel fish not included in statistical analysis. Different letters indicate significant difference.

Histological examination of fish sampled immediately after FW treatment showed that lesions were cleared of amoebae and cellular debris (Figure 5.4a). When fish were sampled after FW treatment, the prevalence of fish with hyperplastic lesions were significantly lower

($P < 0.001$) seven days after treatment and the majority of lesions had recovered as compared to SW and OLE treatment (Figure 5.4b). When fish were sampled immediately after OLE treatment (Figure 5.4c), most lesions were cleared of amoebae, additionally the majority of lesions were undergoing recovery seven days after OLE treatment. When fish were sampled seven days after treatment, lesions in OLE and FW treated fish were recovering (Figure 5.4d) and had significantly smaller ($P < 0.001$) lesion size as compared to SW treated fish (Figure 5.9). The clearance of amoebae and cellular debris was not observed in fish sampled before and immediately after SW treatment (Figure 5.4e). When fish treated with SW were sampled seven days after treatment, fish has significantly larger ($P < 0.001$) lesion size as compared to FW and OLE treated fish (Figure 5.4f).

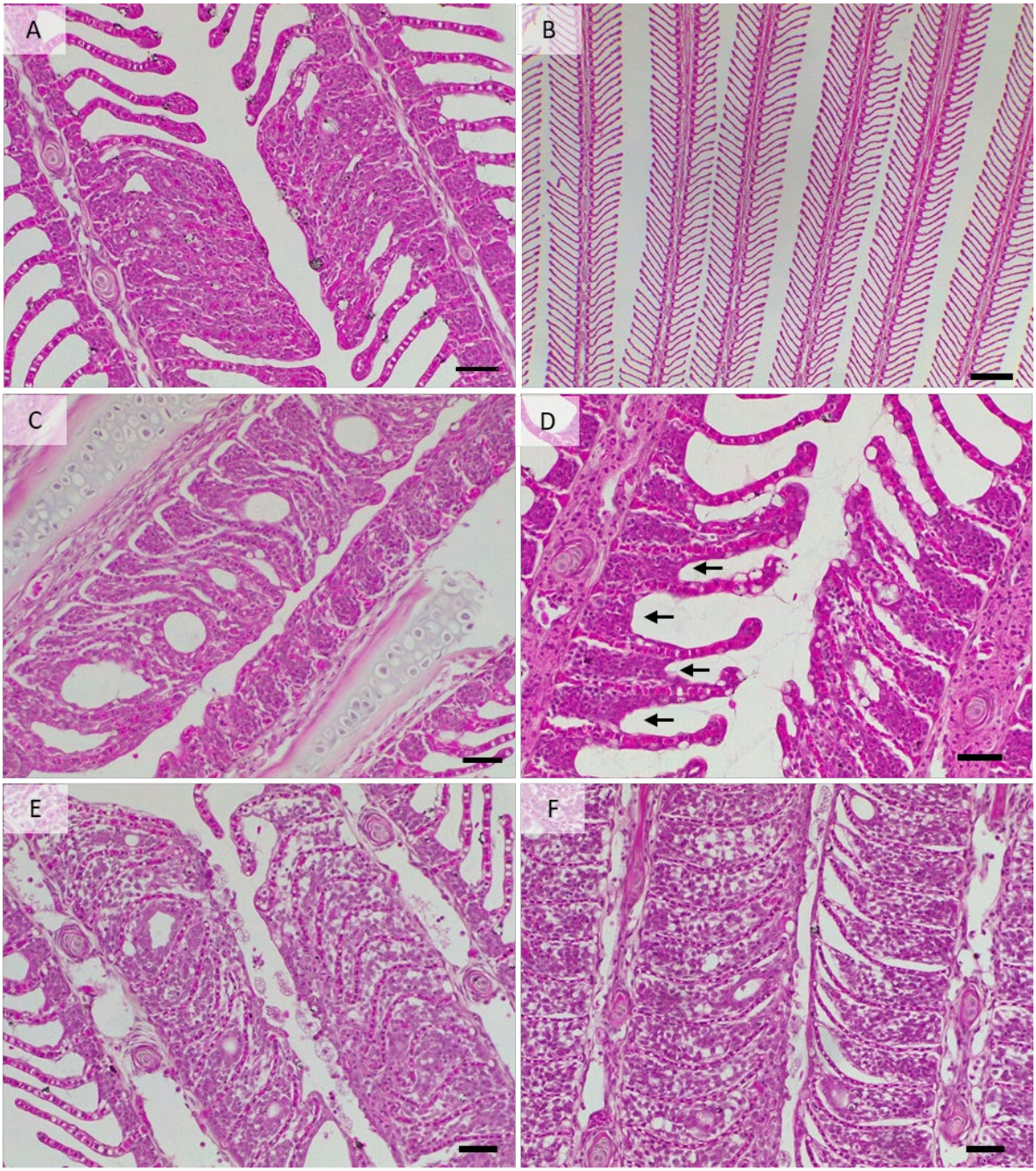

Figure 5.4. Histology of salmon gills immediately after treatment and seven days after treatment. (A) Salmon gill immediately after FW bath; no amoebae were found on gill lesions from fish sampled after bath. Bars = 50 µm. (B) Salmon gill seven days after freshwater treatment; gills appear normal. Bar = 200 µm. (C) . Salmon gill immediately after OLE bath; majority of gill lesions were free of amoebae from fish

sampled after bath. Bar = 50 µm. (D) Gills seven days after OLE bath showing lower prevalence of lamellar fusion and lesion size. Bar = 50 µm. (E) Gill section immediately after SW bath showing trophozoites in abundance upon a lesion surface. Bar = 50 µm. (F) Gill seven days after SW bath with trophozoites present upon lesion surface, increased lesion size and lamellar fusion. Bar = 50 µm.

The prevalence of fish affected by AGD was significantly lower ($P < 0.001$) in fish sampled immediately after OLE and FW treatment as compared to fish sampled before OLE and FW treatment. There was no significant difference ($P > 0.05$) in the prevalence of fish affected by AGD in fish sampled before and immediately after SW treatment. Fish sampled seven days after OLE and FW treatment had a significantly lower prevalence of fish affected by AGD as compared to fish sampled seven days after treatment with SW ($F = 1865.327$, df 2,6, $P < 0.001$). When fish were sampled seven days after treatment, 8.33% ± 2.03 of fish treated with OLE were affected by AGD compared to 100% of fish treated with SW and 0% of fish treated with FW (Figure 5.5).

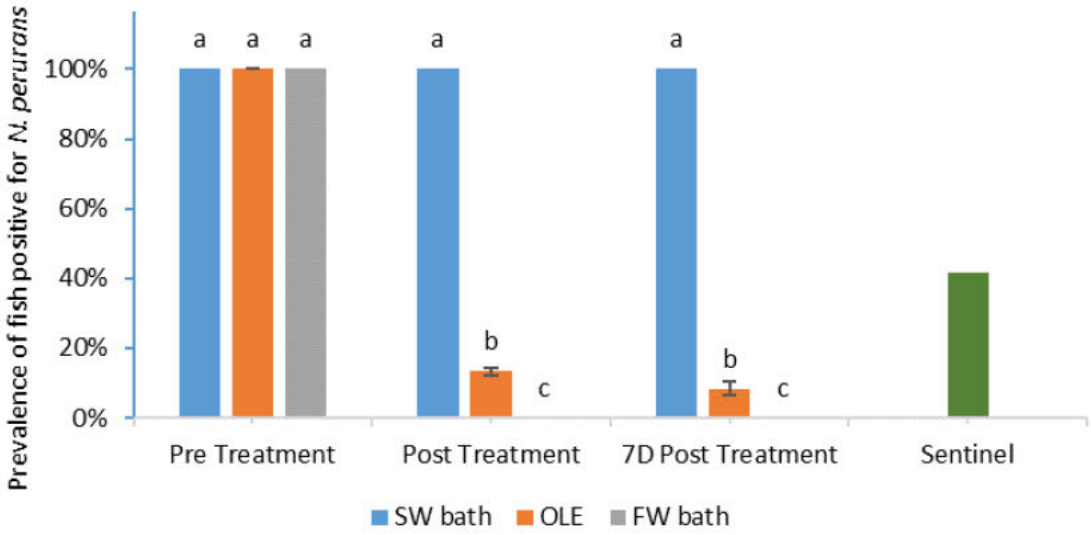

Figure 5.5. Prevalence of fish affected by AGD. Compared against OLE bath, FW bath had better resolution of AGD. Results were presented as means ± SE, sentinel fish not included in statistical analysis. Different letters indicate significant difference.

Across all treatments, the prevalence of fish with hyperplastic lesions was not significantly different ($P > 0.05$) in fish sampled before and immediately after all treatments (Figure 5.6). In fish sampled seven days after treatment, fish that were treated with FW had a significantly lower prevalence of hyperplastic lesions as compared to fish that were treated with SW and OLE ($F = 115.442$, df 2, 6, $P < 0.001$).

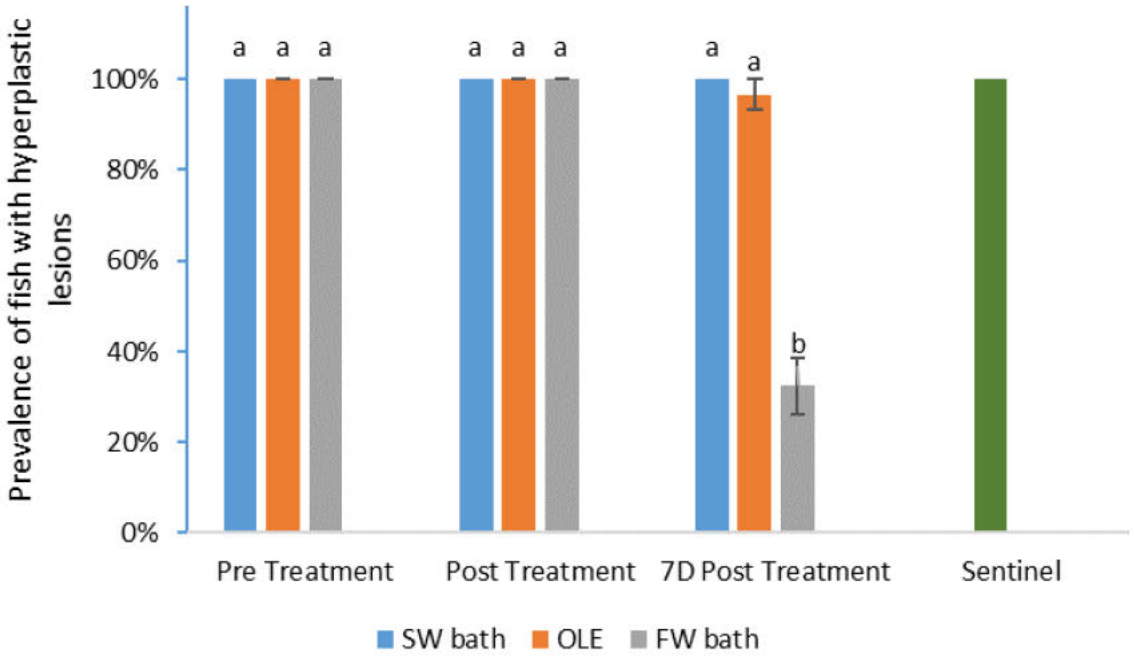

Figure 5.6. Prevalence of fish with hyperplastic lesion. Note Fish treated with FW bath had significantly lower prevalence of hyperplastic lesion 7 days post treatment. Results were presented as means ± SE. Different letters indicate significant difference, sentinel fish (green bar) were not included in statistical analysis.

In fish sampled across all treatment groups, the prevalence of filaments with hyperplastic lesions was not significantly different before and immediately after treatment (F = 18.348, df 2, 156, P <0.001). When fish were sampled seven days after treatment, fish treated with FW had significantly lower (P < 0.001) prevalence of filaments with hyperplastic lesions as compared to fish treated with OLE and SW. When fish were sampled seven days after treatment, fish that were treated with FW had 2.52% ± 0.89 of filaments with hyperplastic lesions as compared to 16.54% ± 1.2 of fish treated with SW and 11.36 ± 1.34 of fish treated with OLE (Figure 5.7). Despite no significant difference in the sampled fish (P > 0.05), fish

treated with OLE had a lower prevalence of filaments with hyperplastic lesions when compared against fish that were treated with SW.

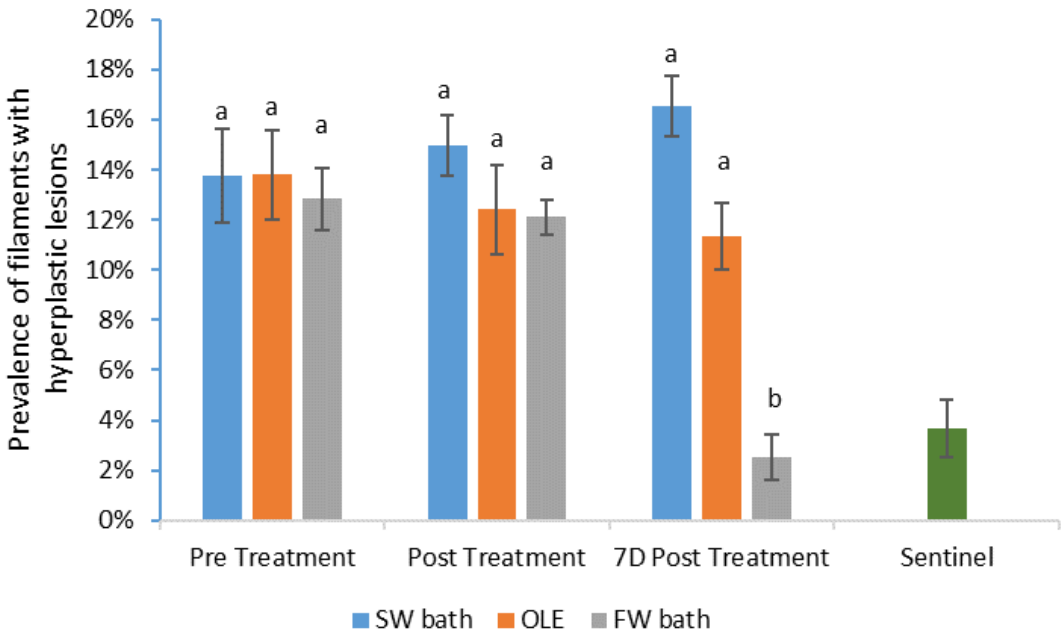

Figure 5.7. Prevalence of filaments with hyperplastic lesion. Seven days after treatment, FW had significantly lower prevalence of filaments with hyperplastic lesion compared to SW bath. Results were presented as means ± SE, sentinel fish not included in statistical analysis. Different letters indicate significant difference.

In fish sampled before treatment, there was no significant difference in the prevalence of lesions colonised with amoebae across all treatment groups (F = 28.769, df 2, 144, P <0.001). When fish were sampled immediately after treatment, fish treated with FW had significantly lower (P < 0.001) prevalence of lesions colonised with amoebae when compared against SW and OLE treatment. When fish were sampled seven days after treatment, fish treated with OLE and FW had significantly lower (P < 0.001) prevalence of lesions colonised with amoebae when compared against fish treated with SW (Figure 5.8).

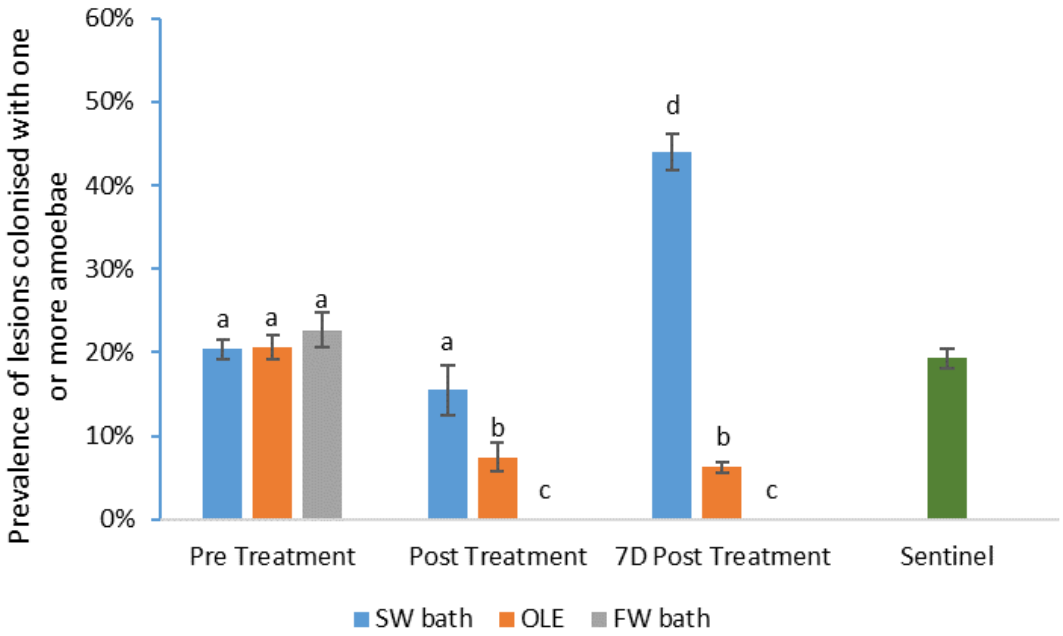

Figure 5.8. Prevalence of lesions colonised with amoebae. Note OLE had significantly lower prevalence of lesions colonised with amoebae as compared to SW bath. Results were presented as means ± SE, sentinel fish not included in statistical analysis. Different letters indicate significant difference.

When fish were sampled before and immediately after treatment, there was no significant difference in lesion size between all treatments (F = 66.643, df 2, 1066, P < 0.001). When fish were sampled seven days after treatment, there was no significant difference (P > 0.05) between fish treated with FW and OLE, fish had a mean lesion size of 5.67 ± 0.25 and 6.55 ± 0.20 interlamellar units respectively. Comparatively in fish sampled seven days after treatment, fish that were treated with SW had significantly larger (P < 0.001) mean lesion size as compared to fish that were treated with SW and OLE (Figure 5.9). When fish were sampled seven days after treatment, fish that were treated with SW had significantly larger (P < 0.001) mean lesion size when compared against fish before SW treatment.

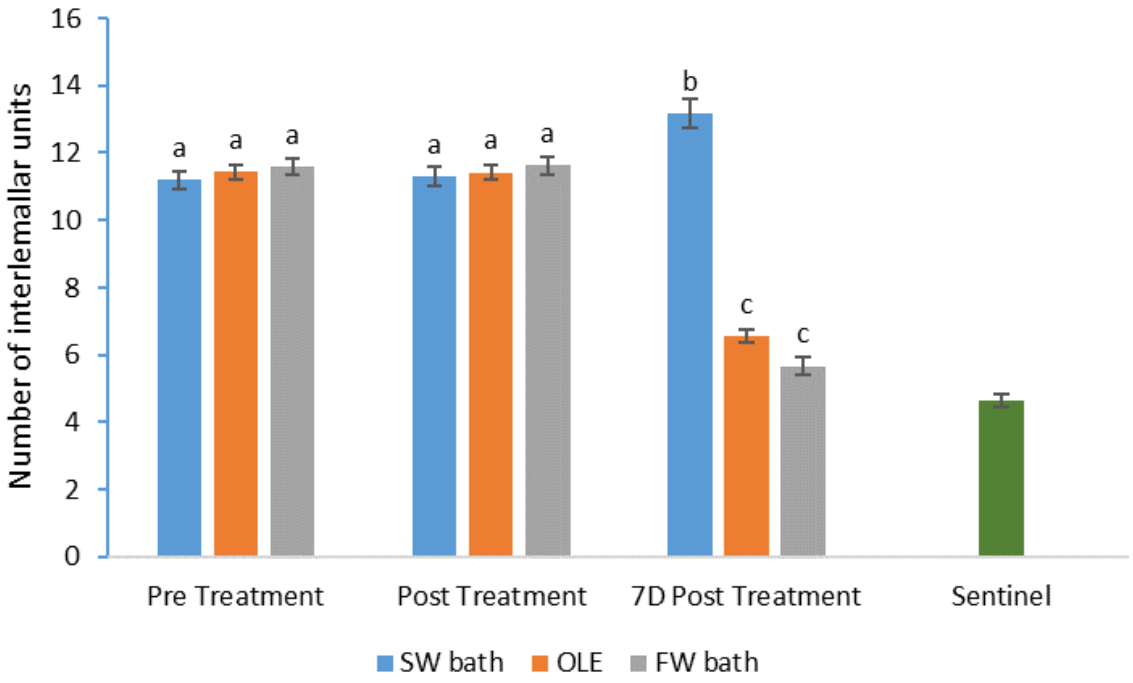

Figure 5.9. Mean lesion size of hyperplastic lesions characteristic of AGD affected fish. Seven days after treatment, both OLE and FW bath had lesions that were smaller compared to SW bath. Results were presented as means ± SE, sentinel fish not included in statistical analysis. Different letters indicate significant difference.

Approximately five minutes after fish were transferred into each OLE bath a mucus like substance was seen floating on the surface. This substance appeared to be stained with OLE. The substance was viscous when rubbed between two fingers. This was not observed in either the FW or SW bath.

Fish in the sentinel group displayed histological signs of AGD seven days after transfer into the experimental system; 41.68% of fish were AGD affected. All fish in this group presented with hyperplastic lesions. On average 3.67% ± 0.51 of filaments presented with hyperplastic

lesions, 1.11% ± 0.41 of filaments with hyperplastic lesions were colonised with amoebae and 19.3% ± 11.64 of lesions were colonised with one or more amoebae.

5.4. Discussion

In this study, the efficacy of OLE as a treatment for experimentally induced AGD was compared against FW. It was shown that OLE was able to significantly reduce the prevalence of AGD affected fish by 82 % immediately after treatment as compared to SW control. This suggests that OLE warrants further investigations as a potential therapeutic agent for AGD. Similarly, OLE was effective against *Leishmania* spp. however, it was observed that different olive cultivar had different efficacy against different species (Sifaoui et al., 2014a). It was found that maslinic and oleanolic acids isolated from OLE were able to induce plasma membrane permeabilization, reduce the mitochondrial membrane potential and decease ATP level in *Leishmania* spp. (Sifaoui et al., 2014c). This is the first time that a plant product had been successfully tested and had significant results *in vivo* on fish with AGD. This experiment indicated that both chemotherapeutants and natural plant products should be considered as a potential treatment against parasitic disease in fish.

The selected concentration of 780 mg L^{-1}, based on *in vitro* results from Chapter 4, did not clear lesions of amoebae as effectively as FW bathing. This resulted in AGD resolution with lower effectiveness as compared to FW bathing. During OLE treatment, fish were observed to be shedding a mucus like substance however, the source of this substance could not be identified. This observation is not exclusive to fish treated with OLE, more likely OLE provided a colour contrast resulting in this observation. Previous study has indicated that during freshwater bathing mucus is released from the gill filaments (Parsons et al., 2001b),

and caused an increase in the number of mucus cells on gills (Powell et al., 2001). This suggests that the mechanisms that clear and kill amoebae are likely to be different between treatments. OLE may act in a manner other than hydration (Powell et al., 2008) to penetrate the mucus layer and subject amoebae to its antimicrobial properties.

In this experiment, OLE was administered topically in a seawater bath and this might have affected the interaction between OLE and trophozoites. When OLE efficacy *in vitro* was determined in Chapter 4 using gill isolated amoebae, the gill mucus associated with the amoebae was removed as part of the isolation process. As fish gills are normally covered by a layer of mucus (Roberts and Powell, 2005a), during *in vivo* bath treatment, gill associated amoebae might be covered by a layer of mucus which hindered contact between amoebae and OLE. This mucus layer might be providing a shielding effect which hindered direct contact between OLE and amoebae. Furthermore, it was observed that the number of gill mucus cells increases in response to AGD suggesting an increase in mucus production (Roberts and Powell, 2005a). This suggests that due to the physiological effects associated with AGD, the efficacy of OLE was affected and that a higher concentration might be needed to achieve AGD resolution similar to FW treatment.

During freshwater bathing, the mucus layer of Atlantic salmon gills is rapidly hydrated which lowers viscosity and osmolality which promotes shedding of the mucus layer (Roberts & Powell 2008). Gill associated amoebae are then exposed to the hypo-osmotic effects of freshwater which causes detachment and lysis. The reduced effectiveness of OLE in this study as compared to Chapter 4 could be due to gill associated amoebae being exposed to a sub-lethal dose of OLE or that the mode of action of OLE is different from FW. When

bacteria were exposed to olive polyphenols, genes for motility, Krebs cycle and cellular respiration were downregulated while genes for DNA repair and DNA recombination were upregulated (Carraro et al., 2014; Li et al., 2016). Along with disruption to gene regulation, changes to cell membrane were observed in scanning electron microscope images after exposure to sub-lethal concentration of OLE (Liu et al., 2017). Olive polyphenols cause downregulation of *fimI*, *flu*, *csgC*, *yddV*, *pgaA* and *rcsA* genes affecting adhesion of cell membrane to surface (Carraro et al., 2014). Furthermore, it was shown at higher concentrations, tea polyphenols required a shorter time in order to lyse cells (Tamba et al., 2007). Therefore, due to its different mode of action, it is likely that in order for OLE to be as effective as FW, treatment duration may have to be extended or concentration increased.

There was no reduction in the prevalence of AGD in fish sampled before, immediately after and seven days after SW treatment. In this experiment, seawater bathing was used as a control to ensure that any effects of OLE were not due to seawater bathing or the mechanical actions associated with fish handling. Similar results were observed in a previous study when the number of gill patches and mortalities of fish in the control group were increasing over seven days (Munday and Zilberg, 2003). This indicated that the progression of AGD in this experiment was not affected by fish handling and other than OLE there were no other variables affecting this experiment.

In this study, there was evidence of cross infection to naïve fish introduced to the system concurrently with treated fish. The modular system used in this experiment was designed with a central sump in which treated water is returned to individual tanks. Similar to the induction of AGD via cohabitation with infected fish in a previous study (Munday et al.,

2001), naïve fish in this study were infected by being held in the same system as diseased fish. Furthermore, the tops of the tanks were not covered and water may have splashed from a tank that held SW treated fish into the tank that held the naïve fish. Despite evidence of cross infection, there were significant differences in AGD indices of fish in different treatment groups. This suggests that cross infection is unlikely to have affected the outcome of this study. Furthermore, a previous study has indicated that it is the fish themselves that are reservoirs of *N. perurans* (González, 2016), and reinfection is largely dependent on remaining live amoebae after treatment (Clark et al., 2003). This indicated that the effects of cross infection were minimal and it did not significantly affect the outcome of this study.

This is the first time that OLE was evaluated as an antimicrobial for treating a parasitic disease in fish. Results indicated OLE as a potential therapeutic drug against AGD in Atlantic salmon. Further studies should be carried out to elucidate the mode of action of OLE and investigate different treatment regimens for efficacy. OLE is a complex mixture consisting of several phytochemical compounds; therefore, it is unknown if the observed activity against AGD is due to a single compound, several compounds or the synergy between the compounds. Future studies should investigate the effects of individual components of OLE in order to better develop it as a treatment.

Chapter Six

General Discussion

Chapter 6 General Discussion

Research showed that olive leave extract (OLE) was efficacious both *in vitro* and *in vivo* against *Neoparamoeba perurans*, the causative agent of Amoebic Gill Disease (AGD) (Crosbie et al., 2012a). This is the first time that OLE or a similar product was tested on Atlantic salmon *Salmo salar* as a treatment for AGD. Plant products are increasingly popular in animal farming as the European Union (EU) had banned the use of antibiotics on farmed animals due to concerns regarding chemical residues and antibiotic resistance (Parrillo et al., 2017). This has led to plant products being studied extensively for its therapeutic, prophylactic, immunostimulating and growth stimulating effects (Makkar et al., 2007). Therapies and prophylaxes with plant products are likely to minimise the risks of antibiotics resistance and chemical residues in fish products (Parrillo et al., 2017). Thus, the development of OLE as a treatment for AGD could have global applications in the future of fish farming.

The anti-parasitic effect of OLE was demonstrated in the current study (Chapter 3, 4 and 5). Bath treatment using OLE significantly decreased the parasitic burden of AGD-infected fish. OLE demonstrated similar antiparasitic effects on other parasites by inhibiting *in vitro* growth of *Leishmania* spp. (Sifaoui et al., 2014a; Sifaoui et al., 2014c) and *Acanthamoeba* spp. (Sifaoui et al., 2013; Sifaoui et al., 2014b). When oleuropein, purified from OLE, was tested on mice infected with *Leishmania donovani*, oleuropein reduced the parasite burden by > 80% after 28 days treatment (Kyriazis et al., 2013). The anti-parasitic effect of OLE persisted for 42 days after treatment which further reduced the parasitic burden by > 95% (Kyriazis et al., 2013). Given that OLE had demonstrated *in vitro* and *in vivo* antiparasitic

properties on different species affecting different hosts, it is hypothesised that the antiparasitic properties of OLE could be efficacious against other fish parasitic disease. Therefore, the antiparasitic effects of OLE could potentially be examined as an alternative treatment against parasites affecting commercial aquaculture such as blood fluke *Cardicola forsteri* (Hardy-Smith et al., 2012) and sea lice (Pike, 1989). Being a natural product, OLE or its derivatives could possibly avoid the negative side effects associated with drugs in aquaculture (Goldburg et al., 2001).

Based on the *in vitro* results of the current study, a high concentration of OLE is required to be effective against AGD. Due to the high concentration required, OLE may be more suitable for use in an experimental system where the volume required for bath treatment is relatively small. As marine salmon production occurs on a much larger scale, it may not be economically viable. Based on a well-boat treatment volume of $300m^3$ (Nilsen et al., 2017) and retail price of $65 per kilogram OLE (Epharmacy, 2018), the cost of OLE for each treatment bath would be approximately $14,625. Comparatively, the cost of freshwater for each freshwater treatment would cost approximately $491 (Taswater, 2018). However, the ultimate goal of any treatment is oral delivery (Ramstad et al., 2002). Small-scale pilot experiment should be conducted to evaluate in-feed safety, followed by assessing the ameliorative effects of OLE on AGD infected fish.

In-feed treatment delivery is advantageous because it allows treatment during bad weather conditions, concurrent application of treatment across all production cages and eliminates stress due to fish handling during bath treatments (Stone et al., 1999). OLE has been successfully delivered to *Pagrus major* by top coating feed pellets using agar as a binder

(Arsyad et al., 2018) or to *Seriola quinqueradiata*, *Sparus aurata* and *Onchorynchus mykiss* by incorporating OLE into the pellet (Oyama et al., 2010; Sicuro et al., 2010b; Sicuro et al., 2010a). Palatability is unlikely to be an issue as OLE has been successfully delivered in-feed with no adverse effect on growth (Oyama et al., 2010; Sicuro et al., 2010b; Sicuro et al., 2010a; Arsyad et al., 2018). However, if palatability of OLE is an issue with Atlantic salmon, it could be addressed by microencapsulation (Mourtzinos et al., 2007; Belščak-Cvitanović et al., 2011; Ganje et al., 2016).

Microencapsulation has been successfully tested on Atlantic salmon and was amenable to intestinal uptake and systemic distribution (Ghosh et al., 2015). Microencapsulation aids absorption of the compound by shielding it from the acid in the stomach, digestive enzymes and bile salts (Marteau et al., 2001) which enabled systemic uptake (Ghosh et al., 2015). Furthermore, microencapsulation prevented leeching of amino acids from microencapsulated diets (Yúfera et al., 2002) and improved storage characteristics of feed post production by preventing lipid oxidation (Heinzelmann and Franke, 1999). Future studies should be done to determine the benefits of using microencapsulated OLE during oral treatment of AGD.

Plant products, clove oil and AQUI-S® are commonly used as fish anaesthetics, including in research during AGD experiments. Despite their extensive usage, the effects of clove oil and AQUI-S® on *N. perurans* have not been determined. *In vitro* results from this study indicated that clove oil and AQUI-S® when used at 40 mg L^{-1} were unlikely to have significant effects on AGD induction and progression in experiments. Similarly, the growth and viability of amoebae were not affected when amoebae were subjected to repeated exposures of AQUI-

S® (Chance et al., 2017). Furthermore, AQUI-S® had no effect on AGD re-infection during freshwater bathing (Adams, 2011). During long term *in vitro* culture of *N. perurans*, it was observed that by day 8 amoebae would detach from the agar substrate and migrate into the seawater overlay (Chance et al., 2017). However, exposing amoebae to AQUI-S® every four days resulted in more amoebae remaining attached (Chance et al., 2017). This increment in the attachment of amoebae could be used to mitigate loss of virulence observed in long term clonal culture of *N. perurans* (Bridle et al., 2015). Thus, AQUI-S® is suitable for use as an anaesthetic for fish involved in AGD experiments as it may help with disease progression without an adverse impact on amoebae.

This study examined a range of semi-automated viability assays for use with *N. perurans*. Results indicated that CellTiter-Glo®, which measures ATP (Lundin et al., 1986; Crouch et al., 1993), was more suitable for *N. perurans* viability testing as compared to the other assays. CellTiter-Glo® was shown to work over a wide range of cell concentrations. CellTiter-Glo® also provides a more accurate cell viability assessment compared to trypan blue vital dye which only indicates cell membrane integrity and not cell viability (Nowak et al., 2004). Therefore, viability assays based on trophozoite metabolic activity are more suitable for use with *N. perurans*. CellTiter-Glo® provides several advantages such as ease of use, high sample throughput, elimination of operator bias and rapid detection of viability (Table 6.1). As a result, CellTiter-Glo® was determined to be a suitable replacement for manual methods.

Table 6.1. Viability tests and the hypothesised time required to perform each viability count. All times shown do not include contact or exposure time for the compound that is being screened. Times estimated are based on the author's experience.

Viability test	Time per viability count	Time per 75 viability count	Advantages	Disadvantages	References
CellTiter-Glo®	5 s to add reagent + 10 min incubation + 2 min for plate reader	5 min to add reagent + 10 min incubation + 2 min for plate reader = 17 min	Quick Easy to use Eliminates operator bias and fatigue	Relatively expensive as compare to manual counting Possible interference with testing compound	This thesis
Neutral red vital dye	~20 – 30 min incubation + ~1 min loading haemocytometer and manual counting	25 min (incubation) + 75 mins (manual counting) = 100 min	Cheap Indicates metabolic activity	Operator error and bias Requires cells to be metabolically active	Crosbie et al. (2007) Crosbie et al. (2014) Chance et al. (2018) Benedicenti et al. (2018) Nowak et al. (2011) Collins et al. (2017) Crosbie et al. (2012a) Adams et al. (2012) Lima et al. (2017)

Viability test	Time per viability count	Time per 75 viability count	Advantages	Disadvantages	References
Trypan blue exclusion dye	~30 s to mix dye with sample + ~ 1 min to load haemocytometer and manual counting	38 min (mixing sample with dye) + 75 min = 113 min	Cheap No incubation required Indicates intact cell membrane	Operator error and bias False positive	Florent et al. (2010) Florent et al. (2009a) Louwen-Skovdam (2008) Leef et al. (2007a) Florent et al. (2007a) Lee et al. (2006) Gross et al. (2006) Villavedra et al. (2005) Morrison et al. (2005) Harris et al. (2005a) Gross et al. (2005) Green et al. (2005)
Most Probable number (MPN) & Growth inhibition assay	14 d incubation time + ~1 min to score wells positive for growth and calculation using MPN algorithm OR growth scoring	> 14 d	Cheap Indicates metabolic activity	Operator error and bias	Howard (2001)

Despite the positive results presented in this thesis, this study had some limitations. Firstly, withholding time and residual levels after treatment with plant products need to be considered (Mitchell, 1988). It is hypothesised that residual levels of OLE are unlikely to be a significant issue for human health as OLE has been used as traditional medicine (Hashmi et al., 2015) and health supplement. However, understanding OLE pharmacokinetics, such as residual levels, is required to establish correct dosage administration for optimal usage of OLE (Birkett, 2002). Fish species, age, water temperature, salinity and method of administration can affect the pharmacokinetics and bioavailability of OLE (Haug and Arnt Hals, 2000). For example, agar-formulated oxytetracycline orally administered to Arctic charr, *Salvelinus alpinus* had higher plasma concentration in a shorter period compared to alginate formulation (Haug and Arnt Hals, 2000). Future studies should examine how these effects affect pharmacokinetics and bioavailability (Birkett, 2002).

OLE is a mixture of compounds (Table 6.2) that varies in composition between different manufacturers (Benavente-Garcıa et al., 2000; Hayes et al., 2011). The lack of standardisation of OLE results in different levels of polyphenols (Table 6.2). By comparing two different sources of OLE (Table 6.2), it can be seen that although OLE from both sources is made up of similar components, the composition can be varied (Benavente-Garcıa et al., 2000; Hayes et al., 2011). Unlike synthesised drugs, i.e. oxytetracycline, OLE is an agricultural product that is affected by factors such as water availability during the growing season (Tovar et al., 2001) and production method (Gimeno et al., 2002). OLE used in my research contains 1.7% oleuropein however, little else is known for this product. Despite repeated approaches, no further information could be obtained from the manufacturer

(Nature's Care Manufacture Pty Ltd). For the development of OLE as a treatment, OLE needs to be standardised for consistency between experiments.

Table 6.2. Phenolic compounds of olive leaf extract used in previous studies (Benavente-Garcıa et al., 2000; Hayes et al., 2011). The values of phenolic compounds are presented as a percentage out of the total extract.

Phenolic Compounds	Guinness Chemical (Ireland) Ltd	Furfural Español S. A. (Alcantarilla, Murcia, Spain)
Oleuropein	40. 33	22. 76
Hydroxytyrosol	1. 82	4. 80
Tyrosol	1. 76	5. 83
Luteolin-7-O-glucoside	5. 05	18. 10
Verbascoside	5. 68	20. 06
Apigenin-7-Oglucoside	3. 13	21. 28

The variation in OLE composition could be due to factors such as growing conditions, harvest time and the difference between cultivars (Kiritsakis et al., 2010; Sifaoui et al., 2013). Previous study has shown that OLE prepared using four different solvents from five different cultivars had varied efficacy against *Acanthamoeba castellanii* trophozoites (Kiritsakis et al., 2010; Sifaoui et al., 2013). The level of polyphenol compounds could be eight times higher in the alcoholic compared to the aqueous extract (Sifaoui et al., 2013). Likewise, variations in growing conditions such as water availability, plant nutrition,

geographical location and time of harvest can influence the composition of the resultant extract (Sudjana et al., 2009a; Lee et al., 2014). For instance, levels of oleuropein and other hydroxyphenyl decreases with lower levels of water irrigation (Tovar et al., 2001). Based on these compositional differences, future studies should be done using individual purified components of OLE or well characterised OLE in order to standardise research.

Only one *in vivo* challenge test was performed to show *in vivo* efficacy of OLE. This means that this thesis did not explore the ameliorative effects of OLE on fish at different temperatures and salinity, fish size, infection status and disease stages (Table 6.3). While it would be advantageous to explore these different parameters as AGD pathogenesis is influenced by different factors (Table 6.3), this was outside the scope of this thesis. It is important to define treatment conditions precisely as some compounds may have a small therapeutic margin (Sommerville et al., 2016). For example, when hydrogen peroxide was used at an effective concentration to treat sea lice, treatment temperatures greater than 20 °C was known to damage fish gills (Thomassen, 1993). Future studies should be done on fish across a wider range of culture conditions at effective concentration and compare treatment efficacy between fish that were previously infected and naïve fish.

Table 6.3. Factors influencing AGD pathogenesis

Factors	Effects	Reference
Fish size	Large fish more resistant to AGD compared to smaller fish	Taylor et al. (2009)
Naïve fish vs re-infected fish	Fish previously exposed to AGD and subsequently challenged were more resistant than naïve fish	Vincent et al. (2006)
Temperature and salinity	AGD development is driven by temperature and salinity	Adams and Nowak (2004)
Treatment temperature	Treatment efficacy of H_2O_2 in seawater is affected by treatment temperature	Adams et al. (2012)

Based on the hypothetical development of OLE as an oral treatment for AGD in Atlantic salmon, my research had fulfilled a substantial part of the requirements needed (Figure 6.1). This four-stage process started with *in vitro* identification of efficacious compounds against the pathogen (Stage 1, Chapter 3). The safety of effective compounds was then determined on the host (Stage 1, Chapter 4). *In vivo* bath efficacy was determined after the compound

was determined to be safe (Stage 2, Chapter 5). From this point onwards, no further bath experiments are warranted as the next stage is the development of oral treatment.

In future experiments, the oral treatment efficacy of OLE and the effects of OLE on palatability and growth will be investigated (Stage 2). Despite previous studies indicating that OLE enriched feed did not affect growth rates of yellowtail *S. quinqueradiata* fed for 42 days (Oyama et al., 2010) and gilthead sea bream *S. aurata* fed for 147 days (Sicuro et al., 2010b). Effects of OLE on growth may not be extrapolated across different species as tolerance levels may vary (Dabrowski et al., 1989; Robaina et al., 1995). Studies have indicated that salmonids are more susceptible to dietary inclusion of plant products such as soybean meal (Dabrowski et al., 1989; Van den Ingh et al., 1991). Inclusion of soybean meal at >25% may induce chronic intestinal inflammation (Burrells et al., 1999), changes to intestinal morphology (Van den Ingh et al., 1991) and reduced growth performance (Dabrowski et al., 1989). However, an OLE enriched diet did not cause inflammation of *O. mykiss* digestive tract despite an increase in docosopentenoic acid which is associated with the inflammatory process (Sicuro et al., 2010b). As OLE is added to feed as an oral treatment, the inclusion level is estimated to be <10% of the diet and unlikely to negatively affect growth (Oyama et al., 2010; Sicuro et al., 2010b).

In Stage 3, the effects of temperature, salinity, fish size and disease status on treatment efficacy will be examined as all these factors can affect AGD pathogenesis (Table 6.3). As *N. perurans* is endemic, the effects of continuous and repetitive long term OLE treatment should also be investigated. Subsequently, the timing of treatment administration should be determined. Previously when fish were treated with bithionol, gross gill score and

prevalence of filaments with lesions were significantly lower ($p < 0.05$) when treatment started two weeks before and continued for four weeks after *N. perurans* exposure (Florent et al., 2007b).

In Stage 4, the efficacy of OLE treatment under field conditions will be evaluated. The efficacy of AGD treatment might be dependent on how often the treatment is administered. Administration of a drug is defined as a period of consecutive days to ensure that the drug has been sufficiently consumed by the maximum number of fish (Sommerville et al., 2016). However, salmonids are known to display hierarchical feeding behaviour which may lead to some fish within the production cage consuming lesser feed (Metcalfe, 1986). Treatment efficacy will depend on fish receiving the full dose of the treatment as medicated feed (Sommerville et al., 2016).

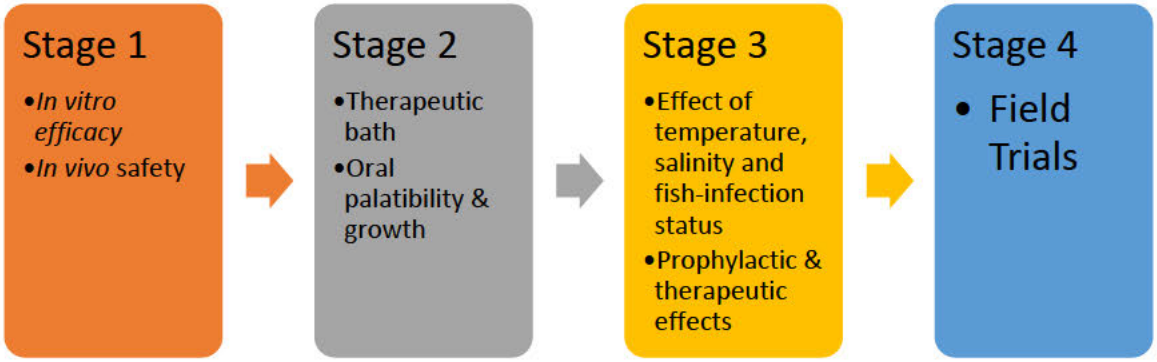

Figure 6.1. Hypothetical development of an oral treatment against AGD.

6. 1 Conclusion

This thesis evaluated the *in vitro* efficacy of several plant products against *N. perurans*. OLE was effective against *N. perurans* both *in vitro* and as a bath treatment *in vivo*. This is the first time that OLE has been used as a treatment for an external parasitic disease affecting Atlantic salmon. Using the proposed hypothetical development of an oral treatment against AGD outlined in Figure 6.1, this thesis had completed a substantial portion of the work required. The results of this thesis indicated that OLE warrants further investigations as a possible in-feed treatment for Atlantic salmon with AGD. Furthermore, future screening studies should be undertaken to discover more plant products with anti-*N. perurans* properties for further investigations. This thesis serves as a proof of concept that plant products with anti-parasitic properties can be effective against a marine parasitic protozoan pathogen.

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Appendix 1

ANOVA table for Percent of filaments with hyperplastic lesions colonised by one or more amoebae

Tests of Between-Subjects Effects

Dependent Variable: Value

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2390.265[a]	8	298.783	43.326	.000
Intercept	3398.049	1	3398.049	492.744	.000
Time_Point	751.036	2	375.518	54.453	.000
Treatment	1133.442	2	566.721	82.179	.000
Time_Point * Treatment	253.249	4	63.312	9.181	.000
Error	1075.803	156	6.896		
Total	6527.710	165			
Corrected Total	3466.068	164			

a. R Squared = .690 (Adjusted R Squared = .674)

ANOVA table for lesion size

Tests of Between-Subjects Effects

Dependent Variable: LesionSize

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5498.254[a]	8	687.282	61.059	.000
Intercept	105667.664	1	105667.664	9387.676	.000
Treatment	1137.020	2	568.510	50.507	.000
Time	1775.291	2	887.646	78.860	.000
Treatment * Time	2973.196	4	743.299	66.036	.000
Error	11998.894	1066	11.256		
Total	140606.000	1075			
Corrected Total	17497.148	1074			

a. R Squared = .314 (Adjusted R Squared = .309)

ANOVA table for Percent of lesions colonised with one or more amoebae (Redo)

Tests of Between-Subjects Effects

Dependent Variable: Value

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5.641[a]	8	.705	45.704	.000
Intercept	8.613	1	8.613	558.232	.000
Treatment	1.611	2	.806	52.213	.000
Time	2.235	2	1.117	72.419	.000
Treatment * Time	1.120	4	.280	18.153	.000
Error	2.222	144	.015		
Total	14.606	153			
Corrected Total	7.863	152			

a. R Squared = .717 (Adjusted R Squared = .702)

ANOVA table for percent of filaments with hyperplastic lesions

Tests of Between-Subjects Effects

Dependent Variable: Value

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1282378.592[a]	8	160297.324	18.348	.000
Intercept	16823483.382	1	16823483.382	1925.663	.000
TreatmentPre	353464.395	2	176732.197	20.229	.000
Time	283149.306	2	141574.653	16.205	.000
TreatmentPre * Time	410973.871	4	102743.468	11.760	.000
Error	1362888.345	156	8736.464		
Total	19281721.013	165			
Corrected Total	2645266.937	164			

a. R Squared = .485 (Adjusted R Squared = .458)

ANOVA table for percent of fish with hyperplastic lesions

Tests of Between-Subjects Effects

Dependent Variable: percentfish

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.286[a]	8	.036	29.703	.000
Intercept	24.864	1	24.864	20656.249	.000
Treatment	.066	2	.033	27.418	.000
Time	.088	2	.044	36.557	.000
Treatment * Time	.132	4	.033	27.418	.000
Error	.022	18	.001		
Total	25.172	27			
Corrected Total	.308	26			

a. R Squared = .930 (Adjusted R Squared = .898)

ANOVA table for percent of fish affected by AGD

Tests of Between-Subjects Effects

Dependent Variable: percentfish

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4.815[a]	8	.602	4438.804	.000
Intercept	13.680	1	13.680	100893.188	.000
treatment	2.110	2	1.055	7780.924	.000
time	1.297	2	.649	4784.201	.000
treatment * time	1.407	4	.352	2595.045	.000
Error	.002	18	.000		
Total	18.498	27			
Corrected Total	4.817	26			

a. R Squared = .999 (Adjusted R Squared = .999)

Appendix 2

Table representation of Figure 2.2

Day	Aqui-S 50 mg L^{-1}	Clove oil 80 mg L^{-1}	Control	Aqui-S 20 mg L^{-1}	Aqui-S 10 mg L^{-1}	Clove oil 40 mg L^{-1}	Clove oil 20 mg L^{-1}	Control
1	28 ± 0.33	27 ± 0.88	27 ± 0.88	26 ± 0.58	26 ± 0.88	27 ± 0.58	26 ± 0.88	27 ± 1.20
2	49 ± 1.45	49 ± 0.33	49 ± 1	47 ± 1.20	48 ± 2.08	48 ± 1.76	47 ± 0.88	51 ±1.33
3	92 ± 0.33	93 ± 2.73	96 ± 2.91	88 ± 2.40	91 ± 3.61	90 ± 1.53	90 ± 0.33	94 ± 3.18
4	180 ± 0.58	182 ± 5.49	186 ± 6.96	172 ± 3.79	176 ± 7.31	175 ± 4	175 ± 1.86	179 5.29±